Structural Characterization and Enzymatic Modification of Soybean Polysaccharides

Pierce, Brian

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Brian Christopher Pierce

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DTU Chemical Engineering

May 2017

Project Supervisors:

Professor Anne S. Meyer (DTU)

Professor Jørn Dalgaard Mikkelsen (DTU)

Jesper Wichmann (DuPont™)

DTU Chemical Engineering
Department of Chemical and Biochemical Engineering

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By
Brian Christopher Pierce

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Preface

The work presented in this thesis was conducted from November 2013 through March 2017 during my time as a PhD student in the Department of Chemical and Biochemical Engineering, Bioprocess Engineering group at the Technical University of Denmark (DTU). This work was conducted as an Industrial PhD project which took place as a joint collaboration between DTU and DuPont™ Nutrition Biosciences ApS with additional funding provided by Innovation Fund Denmark [project number 1355-00146].

This project was supervised at DTU by Professor Anne S. Meyer and Professor Jørn Dalgaard Mikkelsen and at DuPont™ by Jesper Wichmann. I am extremely grateful to my supervisors for their constant support, suggestions, and guidance throughout the entire project. This work would not have been completed without their direction and commitment, and I would like to sincerely thank them for their mentorship. In addition, I would like to thank DTU for providing me with this wonderful opportunity to expand my scientific knowledge and for offering me exposure to so many experts in the areas of biotechnology and carbohydrate chemistry. I would also like to thank Innovation Fund Denmark for providing funding and project assistance to make this idea a reality, and for opening up many opportunities for continued education and growth throughout the project.

This project only became a reality because of the willingness of DuPont™ to invest in fundamental research and embark on this program over three years ago. From the beginning, the success of this project has been due to the efforts of many people within DuPont™. In particular, I would like to sincerely thank Tam Tran for his mentorship, constant belief in me, and never-ending support. It is not an exaggeration to state that this project would never have taken place without his vision and leadership. In addition, I would like to thank Charles Schasteen for giving me my start, continually supporting my education, and always pushing for the highest scientific rigor. I would also like to thank Jørn Marcussen and the entire Advanced Analysis department in Brabrand for welcoming me to Denmark and for providing me with the support needed to undertake this project. In addition, I owe my sincere thanks to the DuPont™ Protein Solutions group, in particular Dan Staerk and Andreas Altemueller, for continuing to support this project in an ever-changing environment. I would like to also thank Tine Vieland Jensen for her help in translating the summary of this thesis to Danish. Lastly, I would like to thank Angela Naef for her strong leadership and continued advocacy of this project from both sides of the ocean.

There have been many other people throughout this project that have been crucial to its success. I would like to sincerely thank Jane Agger for the im-

portant role she played in the analytics and data interpretation of LPMO products during this project. I would also like to thank the group at the University of Melbourne Plant Cell Biology Research Centre, in particular Dr. Tony Bacic, Roshan Cheetamun, and Cherie Beahan, for welcoming me to Australia and for allowing me to learn their techniques for carbohydrate linkage analysis. I would also like to thank NP Analytical Laboratories for their assistance in routine material compositional assays in the early stages of the project.

Finally, I would like to thank our family in the United States who have supported my family and me throughout this entire journey and have continually reminded us that, no matter where we are on the globe, we have a home and family that love us. I would like to sincerely thank my wonderful wife, Allison, for her love and support and for agreeing to come on this adventure to Denmark with me. Lastly, I would like to thank my son, Oscar, for helping me to keep things in perspective and for constantly reminding me of the meaning behind it all.

Brian Christopher Pierce
Kongens Lyngby, Denmark
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Summary

The work in this thesis explores the structure of soybean polysaccharides, and examines approaches for the chemical and enzymatic degradation and solubilization of this material.

Soybean polysaccharides are produced in large quantities globally as a by-product of various soy production processes. The work presented in this text focuses on the insoluble cell wall polysaccharides produced during the manufacture of soy protein isolate. Soybean polysaccharides are water insoluble and feature an approximate carbohydrate composition (by weight) of 35% galactose, 20% glucose, 20% arabinose, 10% galacturonic acid, 8% xylose, 3% rhamnose, and 3% fucose. Currently, the majority of this material is disposed of as waste, increasing production costs. Opportunities exist for the development of novel functional ingredients from this abundant and underutilized material; however, efforts in this area are currently limited by the material's insolubility. A central hypothesis of this work was that by obtaining a more complete understanding of the structure of this material, chemical and enzymatic approaches could be developed to modify the polysaccharides, creating soluble polysaccharide fractions that could provide improved functionality in industrial applications.

To address this hypothesis, structural information was obtained through HPAEC compositional analysis and GC-MS linkage analysis. This work was conducted on the whole soybean polysaccharide fraction, instead of only chemically extracted portions of this material like those analyzed in previous studies. Using this linkage data, the polysaccharide classes in soybean were quantified for the first time, with the results (by weight) identifying the primary constituents as: type I arabinogalactan (27.8%), cellulose (23.5%), (glucurono)arabinoxylan (14.4%), arabinan (8.1%), rhamnogalacturonan I/II (6.2%), xyloglucan (2.7%), type II arabinogalactan (2.0%), and homogalacturonan (1.6%). Using this compositional data, a novel chemical solubilization process was developed utilizing hydrogen peroxide at elevated temperatures. This treatment resulted in the release of more than 70% of the original insoluble material as high molar mass, water-soluble polysaccharides. This solubilized fraction is significantly enriched in the non-cellulosic polysaccharides of soybean such as arabinogalactan, homogalacturonan, rhamnogalacturonan, arabinan, xyloglucan, and (glucurono)arabinoxylan. These results demonstrate that it is possible to solubilize significant portions of the soybean polysaccharide using a one-step chemical treatment, which opens new possibilities for the expanded utilization of this material going forward.

The results from this work also highlight the recalcitrance of soybean cellulose and the significant role that this polysaccharide class plays in the overall insolubility of the material. In an effort to address this, lytic polysaccharide

monooxygenases (LPMOs) were evaluated for their ability to oxidatively degrade soybean cellulose. The initial investigations utilized *Tr*Cel61A, an AA9 LPMO from *Trichoderma reesei*. This enzyme showed no oxidative activity on native soybean polysaccharides; however, significant oxidative degradation was observed on NaOH pretreated soybean polysaccharides. The oxidation products were evaluated using HPAEC and MS, with the results showing oxidation at both the C1 and C4 positions of cellulose. In addition, a synergistic effect between *Tr*Cel61A and a GH5 endo-β-1,4-glucanase was discovered, boosting the glucose release from NaOH pretreated soybean polysaccharides.

Building upon these observations, twenty-three additional LPMOs from seven fungal sources were evaluated (using *Tr*Cel61A as a benchmark), with none showing oxidative activity on native soybean polysaccharides. However, NaOH pretreatment of the raw material was shown to improve the enzymatic accessibility of the soybean cellulose through the removal of non-cellulosic polysaccharides. Following this pretreatment, seven LPMOs (including *Tr*Cel61A) showed activity on the pretreated soybean polysaccharides. These seven enzymes were subsequently evaluated for their ability to increase the glucose release from this material through hydrolytic boosting of endo-β-1,4-glucanase and beta-glucosidase activities. Significant boosting effects were observed for *Tr*Cel61A and one of the newly evaluated LPMOs (Aspte6), resulting in the release of over 36% substrate glucose when compared to only 20% in the absence of the LPMO. Evaluation of the oxidation products from these LPMO treatments with HPAEC and MS showed similar C4 oxidation patterns for all soybean polysaccharide-active LPMOs. In addition, the vast majority of soybean polysaccharide-active LPMOs were also found to have oxidative activity on microcrystalline cellulose. These results demonstrate the ability of enzymatic treatments to solubilize and modify soybean polysaccharides. They also suggest new opportunities to improve upon the enzymatic digestion of this substrate in the future.

Overall, the research conducted in this project has demonstrated the utility of structure-based modification approaches and suggests that the insolubility of soybean polysaccharides is primarily conferred by the cellulosic components. In addition, the results obtained suggest several new opportunities for direct chemical or enzymatic solubilization and degradation of insoluble soybean polysaccharides, paving the way for the improved utilization of this material in the future.

Dansk sammenfatning

Dette studie undersøger strukturen af sojapolysakkarider samt metoder til kemisk- og enzymatisk nedbrydning og opløsning af dette materiale.

Sojapolysakkarider er her defineret som de plantecellevægs-polysakkarider som findes i presseflagerne af sojabønner efter industriel fremstilling af sojaprotein isolat. Sojapolysakkarider produceres på verdensplan i store mængder som et biprodukt fra forskellige andre sojaproduktionsprocesser. Dette studie fokuserer på uopløselige polysakkarider i cellevægen, som produceres under fremstillingen af sojaprotein isolat. Sojapolysakkarider er uopløselige i vand og har en kulhydratssammensætning (i vægt) på ca. 35% galaktose, 20 % glukose, 20% arabinose, 10% galakturonsyre, 8% xylose, 3% rhamnose og 3% fukose.

Størstedelen af dette sojapolysakkaridmateriale ender i dag som affald, hvorved produktionsomkostningerne øges. Denne PhD afhandling er motiveret af ideen om, at der er rig mulighed for at udvikle nye funktionelle ingredienser af denne store mængde overskudsmateriale, men at anvendelserne dog begrænses af materialets uopløselighed. En af hovedhypoteserne i dette studie har været, at man ved at opnå en mere detaljeret forståelse af strukturen af sojapolysakkariderne kan udvikle en kemisk- og/eller enzymatisk metode til målrettet at modificere polysakkariderne, og derved skabe en opløselig polysakkarid fraktion, som kan bruges til at forbedre funktionaliteten i industrielle applikationer.

For at teste hypotesen, er er de strukturelle aspekter (komposition, bindinger) af sojapolysakkariderne undersøgt ved hjælp af GC-MS analyser og HPAEC analyser. I tidligere studier er lignende analyser blevet lavet på kemisk udvundne dele af sojapolysakkarid, men i nærværende PhD afhandling er det for første gang hele sojapolysakkaridfraktionen som er blevet analyseret. De analytiske data blev brugt til kvantitativt at kvalificere polysakkaridklasser af sojabønner. De primære bestanddele var (i vægt) type I arabinogalaktan (27.8%), cellulose (23.5%), (glukurono)arabinoxylan (14.4%), arabinan (8.1%), rhamnogalakturonan I/II (6.2%), xyloglukan (2.7%), type II arabinogalaktan (2.0%) og homogalakturonan (1.6%). Ved hjælp af disse kompositionelle data, blev en ny opløsningsproces udviklet, hvor hydrogenperoxid og opvarmning blev anvendt. Denne behandling resulterede i frigivelsen af mere end 70% af det oprindeligt uopløselige materiale, som bestod af vandopløselige polysakkarider med høj molærmasse. Denne opløselige fraktion viste sig desuden at være rig på de ikke-cellulosiske polysakkarider af sojabønner såsom arabinogalaktan, homogalakturonan, rhamnogalakturonan, arabinan, xyloglukan og (glukurono)arabinoxylan. Disse resultater viser, at det er muligt at opløse en signifikant del af det komplekst sammensatte sojapolysakkaridmateriale ved at anvende en ét-trins kemisk

behandling og denne behandling åbner op for nye muligheder for at udvide brugen af sojapolysakkarider fremadrettet.

Afhandlingens resultater viser at den svært nedbrydelige cellulosedel spiller en stor rolle i den overordnede opløselighed af sojapolysakkariderne. I et forsøg på at afhjælpe denne uopløselighed undersøgtes lytic polysaccharide monooxygenaser (LPMOer) med henblik på at vurdere enzymernes evne til at nedbryde sojabønnecellulose via oxidation. I de tidligere undersøgelser blev *Tr*Cel61A anvendt, som er en AA9 LPMO fra *Trichoderma reesei*. Dette enzym viste ingen oxidativ aktivitet på ubehandlede sojapolysakkarider, dog blev signifikant oxidativ nedbrydning observeret på sojapolysakkarider forbehandlet med natriumhydroxid. Oxidationsprodukterne blev undersøgt med HPAEC og MS, hvor resultaterne viste oxidation på både C1- og C4 positionerne i cellulose. Ydermere blev en synergistisk effekt mellem *Tr*Cel61A og en GH5 endo-β-1,4-glukanase i frigivelsen af glukose fra natriumhydroxid forbehandlede sojapolysakkarider opdaget.

Baseret på disse observationer, blev 23 forskellige AA9 LPMOer fra syv forskellige svampe-kilder undersøgt (og *Tr*Cel61A blev brugt som referenceenzym). Ingen af de 23 LPMOer viste oxiderende aktivitet på de ubehandlede sojapolysakkarider, men på sojapolysakkarider forbehandlet med natriumhydroxid viste syv af LPMOerne aktivitet. Disse syv enzymer blev efterfølgende vurderet på deres evne til at forøge mængden af frigivet glukose fra dette materiale sammen med endo-β-1,4-glukanase og beta-glukosidase. Signifikant forøget effekt blev observeret ved både *TrCel61A* og en af disse nyligt undersøgte LPMO'er (Aspte6), hvilket resulterede i en frigivelse af over 36% glukose fra sojapolysakkariderne sammenlignet med kun 20% uden tilstedeværelsen af LPMO. Undersøgelser af de oxidative produkter fra disse LPMO behandlinger blev foretaget med HPAEC og MS. De viste lignende C4 oxidationsmønstre for alle de otte sojapolysakkarid-aktive LPMO. Endvidere, viste størstedelen af sojapolysakkarid-aktive LPMO'er også oxidativ aktivitet på mikrokrystallinsk cellulose. Disse resulter viser, at enzymatisk behandling kan bruges til at opløse og modificere sojapolysakkarider. Derudover antyder de også nye muligheder for at kunne forbedre den enzymatiske nedbrydning af dette substrat i fremtiden.

Resultaterne i denne afhandling har vist en anvendelighed af at modificere processor baseret på strukturen og antyder at uopløseligheden af sojapolysakkarider stammer fra cellulose. De opnåede resultater danner grundlag for en direkte kemisk- eller enzymatisk opløsning og nedbrydning af uopløselige sojapolysakkarider, hvilket baner vejen for en forbedret brug af dette materiale i fremtiden.

List of publications and patent applications

Publications resulting from this work and included in this thesis:

1. Pierce, B. C.; Wichmann, J.; Tran, T. H.; Cheetamun, R.; Bacic, A.; and Meyer, A. S. (2016). *Formation of water-soluble soybean polysaccharides from spent flakes by hydrogen peroxide treatment*. Carbohydrate Polymers **144**: 504-513.

2. Pierce, B. C.; Agger, J. W.; Wichmann, J.; and Meyer, A. S. (2017). *Oxidative cleavage and hydrolytic boosting of cellulose in soybean spent flakes by Trichoderma reesei Cel61A lytic polysaccharide monooxygenase*. Enzyme and Microbial Technology **98**: 58-66.

3. Pierce, B. C.; Agger, J. W.; Zhang, Z.; Wichmann, J.; and Meyer, A. S. (2017). *A comparative study on the activity of fungal lytic polysaccharide monooxygenases for the depolymerization of cellulose in soybean spent flakes*. Carbohydrate Research **449**: 85-94.

Patent applications resulting from this work:

1. Paullin, J. L., Nambiar, R., Tran, T. H., Pierce, B. C. (2016) US Patent Application No. PCT/US2016/024585.

List of abbreviations

AA	Ascorbic acid
AG	Arabinogalactan
AOAC	Association of analytical communities
BG	Beta-glucosidase
CMC	Carboxymethylcellulose
DP	Degree of polymerization
DQF-COSY	Double quantum filtered correlation spectroscopy
EG	Endoglucanase
ESI	Electrospray ionization
GC	Gas chromatography
GH	Glycoside hydrolase
HG	Homogalacturonan
HILIC	Hydrophilic interaction liquid chromatography
HM	Heteromannan
HMBC	Heteronuclear multiple-bond correlation
HPAEC	High performance anion-exchange chromatography
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum correlation
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
IDF	Insoluble dietary fiber
LC	Liquid chromatography
LPMO	Lytic polysaccharide monooxygenase
m/z	Mass to charge ratio
MALDI	Matrix-assisted laser desorption/ionization
MALS	Multi-angle light scattering
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PAD	Pulsed amperometric detection
PASC	Phosphoric acid swollen cellulose
PGC	Porous graphitic carbon chromatography
RGI / RGII	Rhamnogalacturonan I / Rhamnogalacturonan II
SDF	Soluble dietary fiber
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
SPI	Soy protein isolate
SSPS	Soluble soybean polysaccharides (commercial product - Fuji Oil Co., Ltd.)
TDF	Total dietary fiber
TOCSY	Homonuclear total correlation spectroscopy
TOF	Time of flight
XG	Xyloglucan

Amino acids are identified by their standard 3 letter abbreviations

1. Introduction

1.1 Project Background

Soybeans are a legume oilseed crop produced in large quantities globally. The high oil content, protein levels, and nutritive value of soybeans make them ideally suited for a variety of applications such as the production of oil, animal feed, and human food products. This product flexibility makes soybeans resilient against market fluctuations and has resulted in an increased expansion of global soybean crop production (Grau, Aide, & Gasparri, 2005). Figure 1.1 depicts the increase in both soybean and oilseed global crop production from the 2000/2001 crop year (September 1st - August 31st) until today. United States Department of Agriculture (USDA) projections predict a record year for global soybean production in 2016/2017, with 338 million metric tons produced. This represents a 93% increase in global soybean production since 2000/2001 and illustrates the increased interest worldwide in the production of this versatile crop. While overall crop production is also increasing globally, it is worthwhile to note that soybeans now represent 10.5% of global crop production compared to just 7.8% in 2000/2001.

While it can vary by production conditions and crop variety, soybeans consist of roughly 40% protein, 35% carbohydrates, 20% lipids, and 5% minerals (Cheftel, Cuq, & Lorient, 1985). The carbohydrates in soybean can be classified into two types: soluble oligomers and insoluble polysaccharides. The soluble oligomers consist predominantly of the disaccharide sucrose, the trisaccharide raffinose, and the tetrasaccharide stachyose, and represent roughly one third of the total carbohydrate composition. The remaining two-thirds are comprised of complex cell wall polysaccharides. This fraction, referred to as the soybean polysaccharides in this text and comprising roughly 23% of the total soybean composition, is the primary focus of this thesis.

Soybean polysaccharides are produced in large quantities globally as a by-product of a variety of food manufacturing processes such as the production of soy protein isolate, tofu, and soy milk. When resulting from the production of soy protein isolate, they are typically referred to industrially as *spent flakes*, indicating that the defatted soybean flakes utilized in the production process are now fully extracted of all protein. When resulting from tofu or soy milk manufacturing, the soybean polysaccharides are referred to as *okara* or *soybean curd residue* (SCR). Each year, the Chinese, Japanese, and Korean tofu industries produce roughly 2.2×10^6, 8.0×10^5, and 3.1×10^5 tons of soybean polysaccharides respectively (Li, Lu, Nan, & Liu, 2012; Li et al., 2013). While a portion of this material is sold into lower value applications such as animal feed, the vast majority is disposed of as waste or burned at significant cost to the manufacturer.

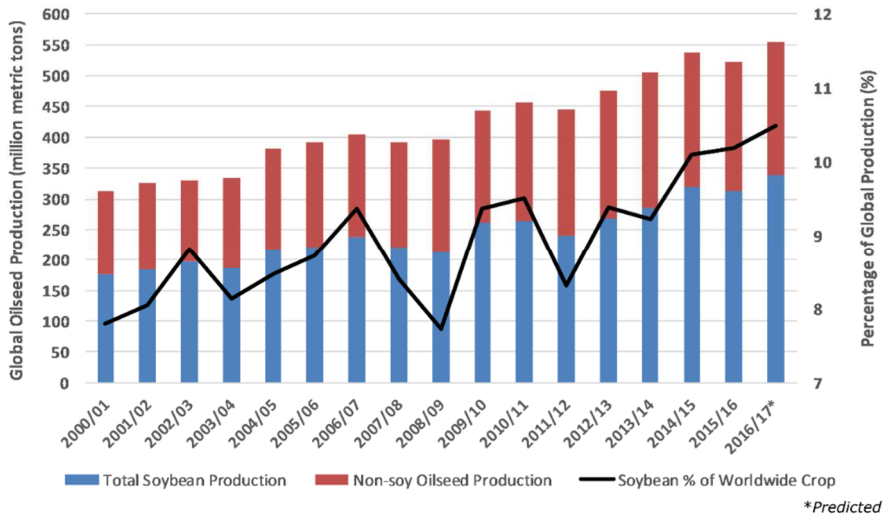

Figure 1.1 - Global soybean and oilseed production for crop years 2000/2001 through 2016/2017 (predicted). Line graph shows the percentage of global crop production represented by soybean. Data compiled by the United States Department of Agriculture, Foreign Agricultural Service Division ("World Agricultural Production," 2016).

For that reason, there is substantial industrial interest in the development of novel applications for this by-product. Currently, soybean polysaccharides generated in the production of soy protein isolate have seen some limited use in the fiber fortification of dry-blended beverages, and as additives to baked goods and extruded snacks. However, soybean polysaccharides present a variety of challenges which have limited their utilization in other applications. The high moisture content of this material presents high costs for drying and shipment, the potential for microbial contamination makes storage challenging, and the lack of water solubility has led to substantial limitations in its utility as a food ingredient. In order to address these challenges, methods for the solubilization and extraction of soybean polysaccharides must be developed. To this end, detailed knowledge of the structure and composition of soybean polysaccharides is required to develop efficient and selective processes to meet this objective. The work presented in this thesis will address these challenges and needs by demonstrating novel techniques for the structural characterization and solubilization of this material.

1.2 Hypotheses and Aims

The overall aim of this project has been to obtain a more complete understanding of the structure, linkages, and chemical composition of soybean polysaccharides and to utilize chemical and enzymatic approaches to modify the polysaccharides for improved functionality in industrial applications. In particular, different modification approaches have been evaluated for their ability to increase the water solubility of this material.

A driving force behind this work has been the industrial desire to create value from soybean spent flakes, an abundant and underutilized industrial by-product from the soy food manufacturing industry. As such, a goal of this work has been to create a larger knowledge base that can be used for processing and utilization of soybean polysaccharides in future applications. The lack of water solubility of this material has severely limited its utilization in many relevant food applications. For this reason, an aim of this project has been to assess different approaches for the degradation and solubilization of this material. With these goals in mind, the following hypotheses were raised to direct the work:

1. Soybean polysaccharides are structurally and chemically unique, and the functionality, notably the insolubility of the native material, is defined by the structural make-up of the carbohydrates.
2. Structural elucidation of soybean polysaccharides will unlock new options for the modification of the material.
3. Targeted chemical treatment and/or enzymatic catalysis can assist in solubilizing the soybean polysaccharides.

In order to further investigate these main hypotheses, three peer-reviewed publications have been completed as part of this project, and for each publication the following additional hypotheses have been raised and addressed:

Paper 1: *Formation of water-soluble soybean polysaccharides from spent flakes by hydrogen peroxide treatment*

a) The carbohydrate composition of soybean polysaccharides is relatively consistent across crop years or manufacturing processes.
b) Chemical treatment can be used to selectively solubilize soybean polysaccharides and to tailor the resulting properties of the soluble products.

Paper 2: *Oxidative cleavage and hydrolytic boosting of cellulose in soybean spent flakes by Trichoderma reesei Cel61A lytic polysaccharide monooxygenase*

c) Pretreatment methods can be utilized to increase the enzyme accessibility of soybean polysaccharides.
d) Oxidizing enzymes are capable of acting on soybean cellulosic polysaccharides, and this activity can improve the enzymatic solubilization of the material by more traditional glycoside hydrolases.

Paper 3: *A comparative study on the activity of fungal lytic polysaccharide monooxygenases for the depolymerization of cellulose in soybean spent flakes*

e) The enzymatic oxidation of soybean polysaccharides is not a universal effect and requires specific enzyme activities.

f) The oxidizing enzymes that display activity on soybean polysaccharides share common traits not present in oxidizing enzymes showing no activity.

1.3 Brief description of the thesis

This thesis intends to describe the considerations, challenges, and approaches taken in the structural characterization and enzymatic modification of soybean polysaccharides. The work has been presented in eleven chapters organized around three peer-reviewed scientific articles. A short summary of the chapter contents is provided below.

Chapter 2 presents a brief overview of cell wall polysaccharide classes and highlights techniques relevant to this thesis for the structural characterization of these materials.

Chapter 3 provides background information on the soybean polysaccharides themselves, outlining how they are produced industrially, presenting a survey of their composition, describing how the material is utilized, and highlighting some of the challenges encountered when working with this material.

Chapter 4 focuses on various approaches for the solubilization of soybean polysaccharides. The work presented centers primarily around chemical solubilization, with enzymatic solubilization covered in subsequent chapters.

Chapter 5 contains a discussion of Paper 1 (the paper is included in the Publications section at the end of this thesis). Paper 1 builds upon the background information in Chapter 3, presenting compositional information for soybean spent flakes sampled over 22 months from industrial soy protein isolate production. In addition, a complete linkage analysis of the constituent polysaccharides in soybean spent flakes is presented. Finally, the work explores the concepts discussed in Chapter 4, presenting a process for the solubilization of soybean spent flakes using hydrogen peroxide.

Chapter 6 presents information on the various approaches for enzymatic solubilization of soybean polysaccharides with traditional glycoside hydrolases. Note that the use of oxidizing enzymes is addressed in the subsequent chapter.

Chapter 7 provides background on the utilization of lytic polysaccharide monooxygenases for the oxidative degradation of polysaccharides. In particular, the applicability of these enzymes to the treatment of soybean polysaccharides is addressed.

Chapter 8 contains a discussion of Paper 2 (the paper is included in the Publications section at the end of this thesis). Paper 2 utilizes the information

presented in Chapter 7 to investigate the activity of the *Tr*Cel61A lytic polysaccharide monooxygenase on soybean spent flakes. It addresses the need for material pretreatment, types of oxidative activity presented by the enzyme, and hydrolytic boosting observed with this enzyme.

Chapter 9 contains a discussion of Paper 3 (the paper is included in the Publications section at the end of this thesis). Paper 3 builds upon the data presented in Paper 2 by exploring the activity of twenty-four unique lytic polysaccharide monooxygenases. The extent of oxidative activity on pretreated soybean spent flakes is assessed and commonalities between those enzymes showing activity are investigated.

Finally, **Chapter 10** presents the main conclusions resulting from the work performed, and **Chapter 11** provides perspectives on the applicability of the results presented within the thesis and suggested directions for future research.

2. Plant cell wall polysaccharides

The goal of this chapter is to present a concise overview of the composition and structures of plant cell wall polysaccharides. While a complete overview of all polysaccharide classes is outside the scope of this work, classes relevant to the discussion of soybean polysaccharides are presented. In addition, key analytical techniques for the structural characterization of these materials are briefly introduced and discussed.

2.1 Polysaccharide classes

Plant cell walls are predominantly composed of polysaccharides, protein, and lignin. Polysaccharides are the most abundant components by far, and can be roughly divided into three distinct polysaccharide groupings: cellulose, hemicellulose, and pectin. Many models have been proposed to explain the organization of these components in the cell wall, often describing a rigid cellulose core which is heavily cross-linked and interspersed with hemicellulose and pectin polysaccharides, forming a complex matrix (Andersson, Westerlund, & Åman, 2006; Keegstra, 2010). Within the hemicellulose and pectin groupings, there exist a wide variety of polysaccharide classes of varying composition and structure. The subsections below highlight the most common classes and those most relevant to soybean polysaccharides.

Soybean polysaccharides contain a significant amount of the monosaccharides galactose and arabinose (further described in Chapter 3). Over the years, the polysaccharides composed of these monosaccharides have been classified as both pectins and hemicelluloses. This section will present these components as part of the pectin fraction, a more representative view of the current understanding of soybean polysaccharide structure (described further in Section 3.3). Starch, which consists primarily of α-1,4-linked glucose units, is also a major plant polysaccharide class. However, starch in soybeans is typically found primarily in germinating soybean cotyledons, with only trace amounts detected in the mature soybean seeds (Wilson, Birmingham, Moon, & Snyder, 1978). For this reason, starch is not included in this summary.

2.1.1 Cellulose

Cellulose is a high molecular weight polymer composed of β-1,4-linked glucopyranose residues. In plant cell walls, cellulose chains have a large molecular weight and can consist of thousands of glucose monomers (Hallac & Ragauskas, 2011). The β-1,4-linked conformation results in chains of glucose where alternating residues are rotated 180° from one another, allowing for significant hydrogen bonding between cellulose chains (Figure 2.1). This results in the energetically favorable formation of large groupings of cellulose chains that are strongly aggregated and have crystalline properties. In nature, these alignments form in a parallel fashion (Type I) but can be converted to

antiparallel alignment (Type II) through strong alkaline treatment (Ochoa-Villarreal, Aispuro-Hernández, Martínez-Téllez, & Vargas-Arispuro, 2012). Moreover, these groupings of cellulose chains also further associate to form a tertiary structure consisting of microfibrils that are associated through hydrogen bonds and van der Waals forces. In this way, cellulose forms a highly self-associated network that is impenetrable to water and highly water insoluble (Robyt, 1998).

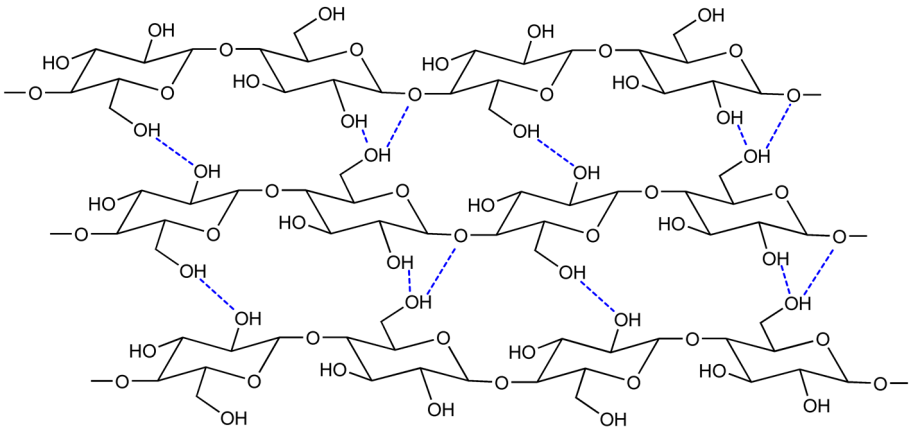

Figure 2.1 - Intermolecular hydrogen bonding (shown in blue) of cellulose chains.

2.1.2 Hemicellulose

Hemicelluloses are a heterogeneous collection of polysaccharides that are lower in molecular weight than cellulose and are heavily associated with cellulose in the cell wall. They share structural similarity to cellulose, consisting of β-1,4-linked backbones; however, they are often heteropolysaccharides having additional monosaccharides linked as sidechains to the polymeric backbone. The three primary types of hemicelluloses are xyloglucans, xylans, and mannans, and each are discussed briefly below.

Xyloglucans

The main backbone of xyloglucan is identical to that of cellulose, consisting of β-1,4-linked glucopyranose residues. However, the glucose monomers are heavily branched with xylopyranose residues linked α-1,6 to the glucan chain. A common repeating unit is typically observed where three consecutive backbone glucose monomers are xylose-substituted, followed by an unsubstituted glucose in the fourth position (the so called XXXG repeating unit). Additionally, some of the xylose sidechains may also be further substituted at the 2-position with a β-galactose residue, which itself may also be substituted at the 2 position with an α-fucose. This additional substitution is particularly common for the substituted residue immediately preceding the unsubstituted glucose in the XXXG repeating unit (Fry, 2010). Xyloglucans strongly associate with cellulose, forming non-covalent interactions that add rigidity and strength to the cell wall. These interactions can be disrupted, but this typically requires the

use of concentrated alkali treatments which are further described in Section 4.1 (Pauly, Albersheim, Darvill, & York, 1999).

Xylans
Xylans consist of a β-1,4-linked xylopyranose backbone with varying sidechain branches. Based on their sidechain substitutions, they can be classified into three primary types: arabinoxylans, glucuronoxylans, and glucuronoarabinoxylans. Arabinoxylans contain α-1,2 and/or α-1,3-linked L-arabinofuranose sidechains; glucuronoxylans contain α-1,2-linked glucuronic acid sidechains; and glucuronoarabinoxylans contain a combination of both substitutions. The glucuronic acid sidechain residues are commonly found in their methylated form (4-*O*-methyl-D-glucopyranosyl uronic acid), and xylose residues can be acetylated at the *O*-2 and/or *O*-3 positions (Robyt, 1998). Xylans are capable of hydrogen-bonding to cellulose; however, these interactions are generally weaker than those observed for xyloglucans (Fry, 2010).

Mannan
The backbone of mannans consists primarily of a β-1,4-linked mannopyranose residues. In addition, some also contain β-glucose residues in the backbone (glucomannan) or 1,6-linked α-galactose residues as side chains (galactomannan) as well. Mannans are present in low abundance in the primary cell wall of dicots and are capable of forming microfibrils in a similar fashion to that observed for cellulose (Robyt, 1998).

2.1.3 Pectin
Pectins are a complex family of polysaccharides containing α-1,4-linked galacturonic acid residues, which can largely be classified into three groups based on their composition: homogalacturonan, substituted galacturonans, and rhamnogalacturonans.

Homogalacturonan
Homoglacturonans are the most abundant component of pectins, comprising roughly 60% of cell wall pectin (Ochoa-Villarreal et al., 2012). They consist of an unbranched chain of α-1,4-linked galacturonic acid residues, which are partially methyl esterified at the uronic acid carboxyl groups and/or acetylated on the *O*-2 and/or *O*-3 position. Methyl esterification of the uronic acid residue results in a loss of charge, and this functionalization commonly occurs in blocks along the homogalacturonan chain. While homogalacturonans are water soluble at neutral and alkaline pH, their solubility is greatly diminished at acidic pH or in the presence of calcium where Ca^{2+} bridges create insoluble cross-linked complexes in the pectin (Fry, 2010). This ionic interaction occurs when greater than 10 unmethyl-esterified residues occur consecutively and coordinate with calcium. This phenomenon, sometimes referred to as the “egg-box model”, is thought to account for the close packing and high-gelling of pectin (Caffall & Mohnen, 2009).

Substituted galacturonans

Two substituted galacturonans have been identified to date: xylogalacturonan and apiogalacturonan. Xylogalacturonan contains the same α-1,4-linked galacturonic acid backbone of homogalacturonan; however, it also contains 1,3-linked xylopyranose residues as sidechains. The size of the xylose side chain varies by source, with xylose monosaccharides or disaccharides being the most common. Interestingly, the longest observed chains (up to 7 xylose residues in length) were identified to be present in the soybean soluble polysaccharides described in Section 3.1 (Nakamura, Furuta, Maeda, Takao, & Nagamatsu, 2002a). Apiogalacturonan is found in the cell walls of aquatic plants and consists of a homogalacturonan backbone, substituted with β-1,2 and/or β-1,3-linked apiose residues (Caffall & Mohnen, 2009).

Rhamnogalacturonans

Rhamnogalacturonans (RG) are polysaccharides that comprise roughly 20-35% of pectin and are traditionally classified into two groups: RGI and RGII (Ochoa-Villarreal et al., 2012). RGI consists of a repeating disaccharide backbone of galacturonic acid and rhamnose in the pattern [→4)-α-D-GalA-(1,2)-α-L-Rha-(1→]. The backbone can be *O*-acetylated at the *O*-2 and/or *O*-3 position of the rhamnose residues. Approximately 50% of the backbone rhamnose residues contain diverse neutral sidechains at the *O*-4 position that are rich in β-galactose and α-arabinose, forming arabinan, galactan, and/or arabinogalactan regions (Caffall & Mohnen, 2009). These extensive neutral sidechain networks allow for the pectin to hydrogen-bond to cellulose (Zykwinska, Ralet, Garnier, & Thibault, 2005). Arabinan consists of α-1,5-linked arabinofuranosyl residues which can be substituted at the *O*-2 or *O*-3 positions with additional arabinofuranosyl residues. Galactan consists of β-1,4-linked galactopyranose units. Arabinogalactans (AG) are grouped into two structurally different types. Type I AG consists of a β-1,4-linked galactopyranose backbone which can be substituted at the *O*-3 position with α-1,5-linked arabinofuranose residues. Type II AG consists of a β-1,3-linked galactopyranose backbone containing β-1,6-linked galactopyranose sidechains that can be further substituted with arabinose, fucose, glucuronic acid, and/or 4-*O*-methyl glucuronic acid residues (Caffall & Mohnen, 2009).

RGII is the most complex branched pectin polysaccharide, but it is a relatively minor component of plant cell walls. RGII has a low molecular weight and a structure consisting of eleven unique glycosyl residues interconnected by over 20 different glycosidic linkages. Unlike RGI, the sidechains of RGII are acidic and contain many unusual residues. The primary structure consists of an α-1,4-linked galacturonic acid backbone that is partially methyl esterified at the C6 position and contains four structurally complex and unique sidechains (Fry, 2010; Ochoa-Villarreal et al., 2012).

2.2 Structural characterization

A variety of analytical techniques exist for the structural characterization of polysaccharides. While it is outside the scope of this thesis to present an exhaustive list, this section will focus on a few key analytical techniques. In general, structural characterization begins with understanding the monomeric building blocks of the polysaccharides. For this project, this was typically accomplished through acid hydrolysis of the polysaccharides and quantification of monosaccharides with HPAEC-PAD (described in Subsection 2.2.1). Following compositional analysis, it is important to understand the various linkages present in the polysaccharide sample. For this project, this was accomplished with GC-MS linkage analysis (described in Subsection 2.2.2). This analysis provides an overview of the constituent polysaccharide classes in the material based on expected linkages and knowledge of known polysaccharide structures. In addition, complementary approaches such as enzymatic structural elucidation or NMR analysis can be used to determine anomeric configurations and perform fine structural elucidation. While NMR analysis was not utilized in this work, it is highlighted in Subsection 2.2.3 due to its widespread use for the structural characterization of carbohydrates. Finally, mass spectrometry was utilized extensively in this project for the identification of oxidations in modified oligosaccharides. This is discussed in greater detail in Subsection 7.3.1 while the utility of this analytical technique is briefly discussed in Subsection 2.2.4.

2.2.1 Monosaccharide analysis

The monosaccharide composition of polysaccharides can be determined through degradation of the polymers to their monosaccharide constituents and subsequent separation, detection, and quantification of these components. The degradation is typically performed through chemical means, with the most common approach being acid hydrolysis using trifluoroacetic acid, hydrochloric acid, or sulfuric acid. The degradation can also be performed enzymatically, with the advantage of this approach being that structural information can be gleaned from the enzyme specificity and the release of specific monosaccharide substituents. Following degradation, high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) can be used to separate and quantify the products (Zhang, Khan, Nunez, Chess, & Szabo, 2012). This separation generally involves a gradient of increasing sodium hydroxide concentration, resulting in early elution of neutral monomers and late elution of acidic monosaccharides due to their highly-retained carboxylic acid functional groups. While HPAEC-PAD was the preferred method for this project, GC or GC-MS analysis can also be used for monosaccharide quantification through conversion of the monomers to alditol acetate derivatives or trimethylsilyl (TMS) derivatives (Blakeney, Harris, Henry, & Stone, 1983; Doco, O'Neill, & Pellerin, 2001).

2.2.2 Linkage analysis

Carbohydrates are constructed of monosaccharides which are connected together through glycosidic linkages. Unlike with proteins or nucleic acids, each monomeric unit is capable of forming linkages at a variety of sites, resulting in complex, heterogeneous structures. As described in Section 2.1, the monosaccharide composition and types of linkages can vary widely across polysaccharide classes and have a significant impact on the resulting properties of the material. This diversity presents an analytical challenge; however, knowledge of these linkages is vital information for understanding the structure and properties of the resulting polysaccharides. Linkage information can be obtained through GC-MS methylation analysis. For samples which contain uronic acids, such as the soybean polysaccharides described in this thesis, additional steps are required to obtain linkage analysis information for these residues (Pettolino, Walsh, Fincher, & Bacic, 2012). The key steps of the procedure are briefly described below, and application of this technique to soybean polysaccharides is further described in Paper 1.

Prior to analysis, samples must be enriched in cell wall polysaccharides by removal of proteins, lipids, phenols, and free sugars which can interfere with the analysis of the polysaccharides. A variety of extractions exist for this purpose and alcohol-insoluble residue (AIR) preparations have been shown to work very well for cell wall polysaccharides (Andersson et al., 2006). One advantage of the AIR procedure over other aqueous buffer extractions is the lower probability of losing water-soluble cell wall polysaccharides. Following the AIR preparation, starch in the sample should be removed through α-amylase treatment. If the linkage analysis samples contain starch, it will complicate the interpretation of the linkage data as starch contains t-glucose, 1,4-glucose, and 1,4,6-glucose linkages. These different linkage types can also be present in other polysaccharide classes frequently encountered in cell wall analysis such as xyloglucan, glucomannans, galactomannans, cellulose, and β-glucans.

Following the sample preparation, the first step in the analysis is to reduce all uronic acids and carboxylic esters present in the sample. Glycosidic linkages adjacent to uronic acids are resistant to hydrolysis, and pretreatment is required to convert these uronic acids so that they resemble their neutral sugar counterpart and are captured following hydrolysis. A two-part reduction is performed utilizing sodium borodeuteride and sodium borohydride, both of which add unique chemical identifiers allowing for the neutral sugar, the uronic acid, and the methyl ester to be distinguished in the linkage analysis (Pettolino et al., 2012). A disadvantage of this procedure is that it requires duplicate analysis for each sample, effectively doubling the number of samples to be analyzed.

Next, the samples are methylated to convert all free hydroxyl groups in the sample to methoxy groups. This procedure chemically labels the hydroxyl groups which were not involved in linkages and will allow these groups to be

distinguished from those involved in linkages following hydrolysis of the polysaccharide to its monosaccharide constituents. This is a critical step in the analysis, and the procedure is typically performed twice, particularly for cell wall polysaccharides, in order to ensure that all free hydroxyl groups are methylated. Following methylation, the polysaccharides are hydrolyzed in 2M trifluoroacetic acid, a volatile acid which can be evaporated away following the reaction. The resulting monosaccharides are then reduced with sodium borodeuteride to permanently open the sugar rings (through reduction of the anomeric carbon carboxyl group to an alcohol) and to deuterate the anomeric carbon for easy identification with MS. It should be noted that this treatment results in a loss of all information about the anomeric configurations (α or β) of the monomers. Following the reduction, the samples are acetylated with acetic anhydride, replacing all free hydroxyl protons with an acetyl group and thereby increasing the volatility of the monosaccharides.

The partially methylated alditol acetates (PMAAs) formed in the previous steps can then be analyzed with GC-MS using a high-polarity column for separation. The retention times for specific PMAAs are highly reproducible for a given column run under identical conditions. The majority of peaks can be assigned to a PMAA based on the relative retention time and the mass spectrum; however, MS-MS fragmentation can be used to identify unknown PMAAs as well. Once all peaks have been identified, the mole percentage of each PMAA is calculated based on the areas of the peaks. By applying knowledge of the composition and linkages of known polysaccharide classes (described in Section 2.1), it is possible to classify and quantify the types of polysaccharides present in the sample (Pettolino et al., 2012). It should be noted that it is not uncommon for this type of approach to result in 10-15% of the observed linkages unassigned. This is an expected result due to the inherent inhomogeneity in plant cell walls and highlights that this characterization should only be viewed as a broad classification of the material.

2.2.3 NMR

Nuclear Magnetic Resonance (NMR) is a phenomenon exhibited by certain atomic nuclei when placed in a static magnetic field and exposed to an additional oscillating magnetic field. NMR active nuclei such as ^{1}H, ^{13}C, or ^{31}P are capable of absorbing electromagnetic radiation at a characteristic frequency. Following excitation at this frequency, the resulting resonance response can be recorded. Finally, a Fourier transform can be utilized to convert the time-domain recorded response into the frequency domain, where information can be obtained about the chemical composition of the sample. NMR spectroscopy offers a wide variety of techniques and methods for the structural assignment of complex carbohydrates (Duus, Gotfredsen, & Bock, 2000). While carbohydrates have characteristic parameters that can be captured with NMR, the structural diversity and heterogeneity of samples makes NMR analysis of complex polysaccharides challenging. Some of the most useful techniques for carbohydrate structural characterization are briefly outlined below.

- One-dimensional ^{1}H and ^{13}C NMR can be used to identify the number of sugar residues in a polysaccharide through investigation of anomeric signals.
- Homonuclear Total Correlation Spectroscopy (TOCSY) allows for observations of long range proton couplings. This property allows determination of all correlations between protons within the same spin system and is particularly useful for oligosaccharides.
- Double Quantum Filtered Correlation Spectroscopy (DQF-COSY) provides information on the ^{1}H-^{1}H couplings in the compound, and thorough interpretation of this spectrum allows determination of the protons that are within 3 bonds of a given proton.
- Heteronuclear Single Quantum Correlation (HSQC) allows for the transfer of proton assignments from the TOCSY and DQF-COSY spectra to carbon atoms, and thereby allows determination of the δ_C chemical shift for each proton.
- Heteronuclear Multiple-bond Correlation (HMBC) allows visualization of 2-4 bond carbon-proton correlations. In addition, these correlations are expressed across heteroatoms such as O.

In general, TOCSY, DQF-COSY, and HSQC can be utilized to trace the proton-proton connectivity within an individual monosaccharide. HMBC can then be used to determine how the individual monosaccharide residues are connected. However, data interpretation can be quite complex, and this complexity greatly increases for less homogeneous substrates and for reaction systems containing mixtures of products. For this reason, NMR structural characterization must be utilized in conjunction with linkage analysis and other analytical techniques for complex polysaccharide samples.

2.2.4 Mass spectrometry

Mass spectrometry (MS) is an analytical technique which separates ions based on their mass-to-charge ratio (*m/z*). In this technique, the molecules in a sample are first ionized to create charged species, which are then accelerated to have identical kinetic energies. These ions are then deflected in an electric or magnetic field, with the lighter and more highly charged masses deflecting to a higher extent. The ions are separated based on this principle and are subsequently detected, with the results giving the abundance of each identified mass-to-charge ratio in the sample. These results can then be correlated with known masses of chemical compounds to identify the components of a given sample.

As demonstrated by the linkage analysis in Subsection 2.2.2, MS is a powerful technique for the characterization of polysaccharide structural information. Some significant advantages of MS-based analyses are the high sensitivity in data collection and the low sample quantities required for analysis. This high sensitivity is particularly useful for mixtures of carbohydrates, where the compounds of interest may be present in low abundance. In order for MS analysis

to be used, polysaccharides must first be converted into smaller oligomers using chemical hydrolysis or enzymatic treatment. As described in Chapter 6, enzymes which specifically cleave certain linkage types can be used in combination with MS analysis to discern structural information.

A significant advantage of MS is the ability to utilize MS^2 or MS^n approaches for the fragmentation of oligosaccharides. These fragmentations can occur at the site of the glycosidic linkages or across the monosaccharide rings, resulting in unique fragmentation patterns which contain a wide variety of structural information. Other common approaches for the MS analysis of carbohydrates involve derivatization to discern information about the structure (see Subsection 2.2.2), use of isotopic labeling to identify structural components, and/or modification of the compound charge state to affect ion intensities (Bauer, 2012). As MS analysis is based on compound masses, it can be a challenge to differentiate carbohydrate classes of identical masses in a heterogeneous mixture. As many carbohydrates feature identical masses but important differences in chemical properties, it is important to employ labelling, enzymatic treatment, or pre-MS chromatographic separation in order to differentiate these compounds. MS analysis was used extensively in this project for the characterization of oxidized oligosaccharides, and this is discussed in greater detail in Subsection 7.3.1, Paper 2, and Paper 3.

3. Soybean polysaccharides: Production, composition, and utilization

The aim of this chapter is to introduce the industrial processes by which soybean polysaccharides are produced and describe the overall composition of this material. The properties of soybean polysaccharides and a concise yet thorough review of prior research on the chemical composition of this material are provided. In addition, current and potential applications for soybean polysaccharides are discussed. Finally, the specific challenges of working with this material are described.

3.1 Production processes

Soybean polysaccharides are produced as an industrial by-product of the manufacture of soy food ingredients such as soy protein isolate, tofu, and soy milk. While the production processes for these products vary, it is important to note that each results in a side-stream enriched in the cell wall polysaccharides from soybean. In all cases, this side-stream is considered a by-product of the production process, and increased utilization of this material would serve to improve the overall process economics.

The production process for soy protein isolate is shown in Figure 3.1. This process begins with the removal of the seed coat (hulls) through cracking and aspiration. This is followed by sample heating to coagulate proteins for improved oil extraction, and hexane extraction to remove the soybean oil. Following hexane removal, the resulting product, referred to as white flakes, is milled and extracted at neutral or alkaline pH to solubilize the soy protein. This stream is subsequently separated by centrifugation, resulting in a solid waste stream referred to as *soy fiber* or *soybean spent flakes* - the fraction containing the soybean polysaccharides. The remaining soluble fraction is then acidified, to induce isoelectric precipitation of the proteins, and separated by centrifugation. The resulting products of this separation are the soy protein isolate itself and the soy whey, a soluble fraction containing small soluble sugars, minerals, and proteins which did not undergo isoelectric precipitation at acidic pH (Lusas & Riaz, 1995). Note that the process described in Figure 3.1 is highly generalized. Industrial processes may involve the use of additional production steps or recycling of downstream process streams to improve protein extraction yields.

From an overall process perspective, the production of soy fiber/soybean spent flakes occurs relatively late in the process (i.e. downstream), as can be observed in Figure 3.1. However, it should be noted that the protein extraction process from soy white flakes is not traditionally conducted in the same facility conducting the oil extraction. For soy protein isolate manufacturers, the initial

protein solubilization from white flakes and separation to remove the spent flakes is the first step in the process. For this reason, the overall material composition of the spent flakes remains relatively consistent, regardless of the various downstream processing techniques being utilized in the protein isolate process.

Figure 3.2 details a generalized production process for soy milk and tofu (Shurtleff & Aoyagi, 2000). In both cases, the primary soybean polysaccharide process stream is the *okara*, the insoluble cell wall polysaccharides separated from the soy milk. This process features a large difference when compared to the soy protein isolate production process depicted in Figure 3.1. Namely, the soybean oil is not directly extracted and instead goes primarily into the soy milk during the first separation step. For this reason, okara features higher levels of fat and protein when compared with the spent flakes produced during soy protein isolate production. The process depicted in Figure 3.2 is the traditional Japanese method for soy milk and tofu manufacturing. In the Chinese method, the cooking step is applied following soy milk separation from the okara residue (Chang, 2006).

Table 3.1 provides a comparison of the representative protein, mineral, fat, and carbohydrate compositions for both okara and soybean spent flakes. The processes used to produce soy milk or tofu can vary significantly, and a range of compositions has been reported in the literature (Li et al., 2013). The okara values in Table 3.1 were chosen from a representative sample collected by Li, Lu, Nan, & Liu (2012), while the values for soybean spent flakes were taken from the representative spent flake sample selected in the research for this thesis and further described in Section 3.2. From the data in Table 3.1, it is clear that the relative protein and fat content of okara is significantly higher than that observed for soybean spent flakes. This result is expected, of course, due to the lack of oil extraction present in the soy milk/tofu process and the efficient protein extraction performed in the soy protein isolate production process.

Despite the compositional differences of these streams in terms of protein, fat, and minerals, the overall composition of the structural polysaccharides is highly conserved, regardless of the production process employed. For this reason, studies conducted on either spent flakes or okara are applicable to both side-streams. For this PhD project, soybean spent flakes were selected as the primary substrate for further investigation and analysis. This is because the spent flakes represent a substrate more highly refined in the structural polysaccharides of soybean. As the primary goal of this research has been to investigate the composition, structure, and accessibility of these complex polysaccharides, this material is more ideally suited for this research. However, it should be noted that the generalized findings around the structures and chemical and enzymatic accessibility of this material can be broadly applied to both substrates.

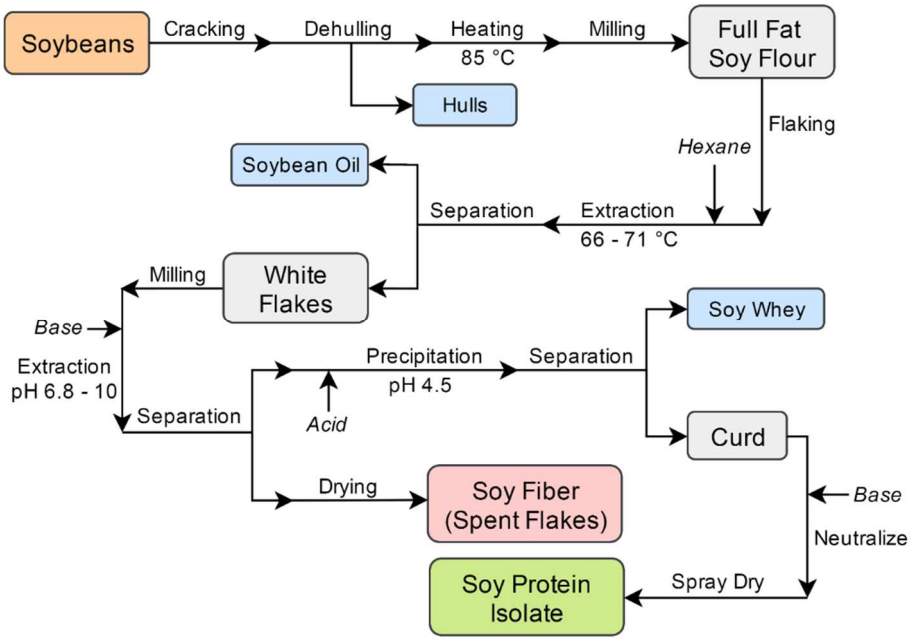

Figure 3.1 - Generalized production process for soy protein isolate (Lusas & Riaz, 1995). The starting soybeans are colored orange, intermediates are colored grey, secondary products are colored blue, the final product is colored green, and the stream containing the soy polysaccharides – soy fiber/spent flakes – is colored pink.

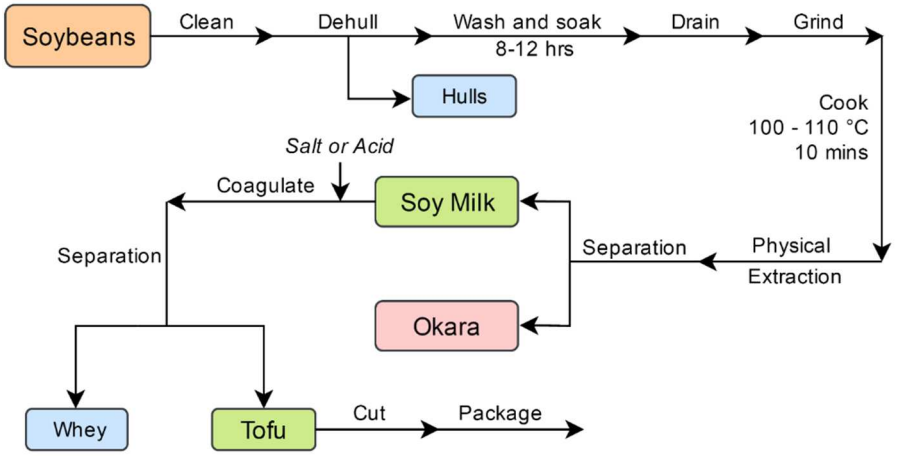

Figure 3.2 - Generalized Japanese production process for soy milk and tofu (Shurtleff & Aoyagi, 2000). The starting soybeans are colored orange, secondary products are colored blue, final products are colored green, and the stream containing the soybean polysaccharides – okara – is colored pink.

Table 3.1 - Representative composition of soybean spent flakes and okara on a dry basis. Okara data was collected from Li, Lu, et al. (2012), and soybean spent flake data is taken from the data presented in Section 3.2 and Paper 1 from this thesis (Pierce et al., 2016).

Material	Protein (%)	Fat (%)	Minerals (%)	Carbohydrates (%)
Soybean spent flakes	10.1	0.8	3.3	85.8
Okara	19.1	6.3	4.2	70.4

3.2 Material composition

As described in Section 3.1, a representative soybean spent flake sample was chosen for the analysis performed in this project. This section will detail the monosaccharide, protein, and mineral composition of this material. In addition, microbial levels are also discussed. While this section will focus only on monosaccharide content, a more detailed look at the polysaccharide composition is presented in Section 3.3 and in Paper 1.

3.2.1 Monosaccharide composition

Soybean polysaccharides are predominantly composed of eight monosaccharides, with four of these comprising roughly 86% of the total monosaccharide content. Figure 3.3 shows a typical monosaccharide breakdown for soybean spent flakes as measured by the HPAEC-PAD method described in Subsection 2.2.1. Galactose, arabinose, glucose, and galacturonic acid are the predominant monosaccharides; however, xylose, rhamnose, fucose, and low levels of glucuronic acid are also present. Note that many studies also report the presence of mannose in soybean polysaccharides at levels ranging from 1.3-3% (Huisman, Schols, & Voragen, 1998; Li, Lu, et al., 2012; Li et al., 2013; Yamaguchi, Ota, & Hatanaka, 1996). However, it has been demonstrated by Ouhida et al. that the mannose content drops significantly when cell wall polysaccharides are isolated after removing other soluble components (Ouhida, Pérez, & Gasa, 2002). The vast majority of the mannose content in soybean is preset in the soybean hulls in galactomannans, which have been removed prior to oil and protein extraction as described in Section 3.1 (Aspinall & Whyte, 1964). In the analysis of soybean spent flakes for this project, only very low levels of terminal mannose were detected with GC-MS linkage analysis (0.2%), and it was not recognized as a primary constituent of any identified polysaccharide classes. For that reason, it has not been included in Figure 3.3. Glucuronic acid is present at extremely low levels in soybean polysaccharides (roughly 0.1%); however, it has been included in Figure 3.3 due to its presence in the reported (glucurono)arabinoxylan polysaccharides of Paper 1.

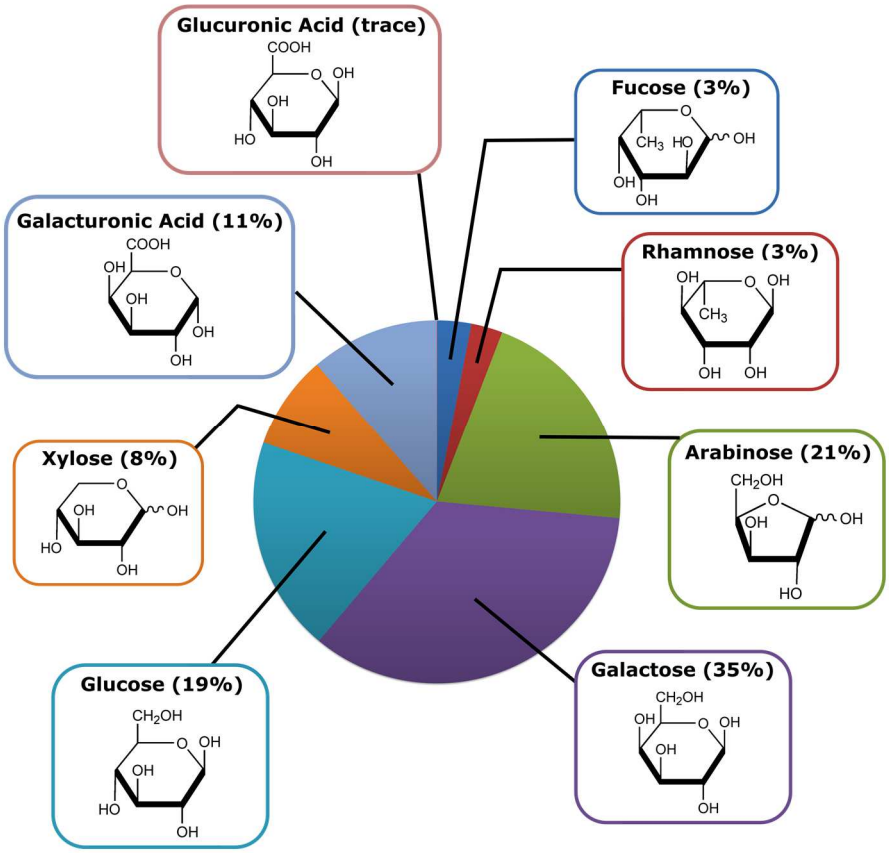

Figure 3.3 - Monosaccharide profile of a representative soybean spent flake sample. Analysis by HPAEC-PAD as reported in thesis Paper 1 (Pierce et al., 2016).

3.2.2 Protein

While the focus of this project has been on soybean polysaccharides alone, soybean spent flakes contain approximately 10% protein that is not extracted during the process shown in Figure 3.1. As it is quite common for the soybean flakes to undergo multiple protein extractions for increased production yields, the protein remaining with the spent flakes is heavily entrenched and cannot be easily removed with conventional alkaline solubilization. Figure 3.4 shows an SDS-PAGE gel for soy protein isolate (SPI) and soybean spent flake (SF) samples collected from 2007-2014. The protein content of the soybean spent flakes shares similarities with the proteins typically extracted as SPI, such as the three bands present from 80-97 kDa: γ-conglycinin, the β-conglycinin α-subunit, and the β-conglycinin α'-subunit. However, the most intense bands are observed around 12 kDa and 31 kDa, which may represent Bowman-Birk protease inhibitor (BBI) and lectin respectively. These results indicate that a portion of the proteins present in the spent flakes differ from the SPI proteins. It is interesting to note the consistency in the protein profile of soybean spent

flakes across the collection window, indicating that residual protein in this material is not simply uncaptured by the SPI process but also highly associated with the soybean polysaccharides.

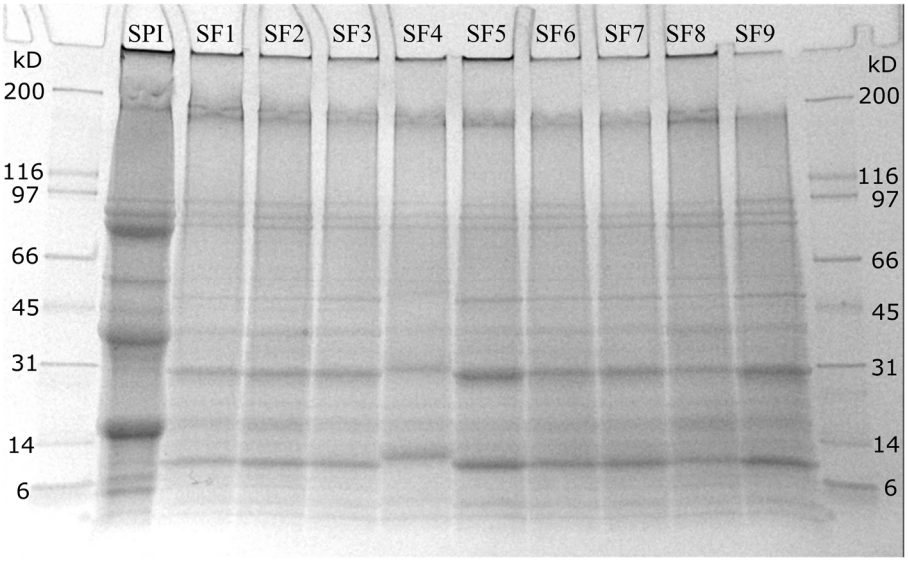

Figure 3.4 - SDS-PAGE gel of soybean spent flakes with molecular weight standards in the first and last gel lanes (kD = kilodalton). SPI is soy protein isolate (DuPont™ SUPRO® 500E) and SF1 through SF9 are soybean spent flake samples collected over various processing years: SF1 (2007), SF2 (2010), SF3 (2009), SF4 (2009), SF5 (2009), SF6 (2011), SF7 (2012), SF8 (2013), SF9 (2014).

These differences are further confirmed through the amino acid compositional analysis presented in Figure 3.5 as measured by AOAC method 982.30 E(a,b,c) (2006). While the overall amino acid compositions are relatively consistent between the SPI and the spent flakes, it is apparent that the spent flakes contain significantly less Glx (glutamine + glutamic acid) and Arg when compared to the SPI. To offset this difference, the spent flakes contain higher levels of Thr, Pro, Gly, Ala, Cys, Val, Met, Leu, and Phe. While further characterization of the protein content of soybean spent flakes was outside the scope of this work, it is worthwhile to note the apparent differences between the protein entrapped in the soybean spent flakes and the proteins typically extracted in the SPI process.

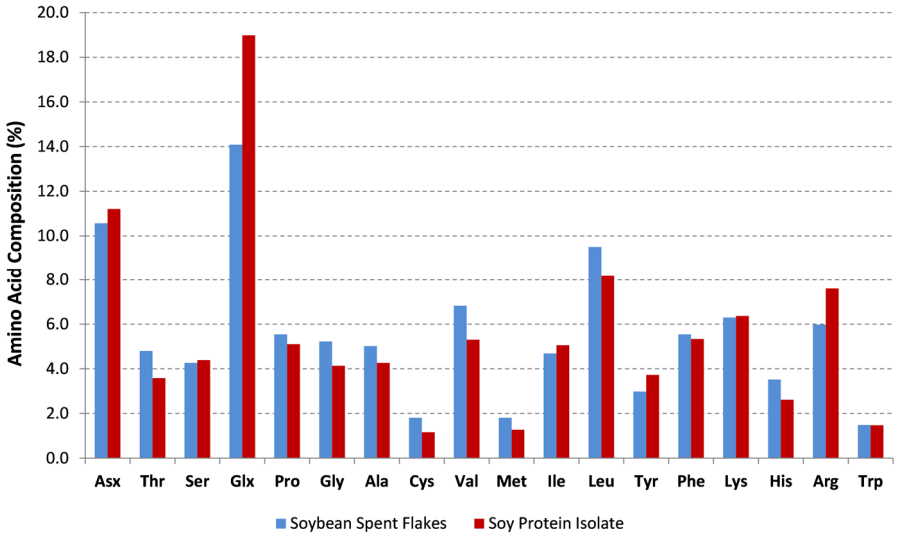

Figure 3.5 - Amino acid profile (percentage of each amino acid based on w/w % measurements) for representative samples of soybean spent flakes and soy protein isolate. Asx represents total asparagine and aspartic acid, and Glx represents total glutamine and glutamic acid. Results shown for triplicate analysis (n=3) with standard deviations < 1.0% for all amino acids. Measured by AOAC method 982.30 E(a,b,c) (2006). Soybean spent flake total protein is 10.1%, and soy protein isolate total protein is 90.4%.

3.2.3 Minerals

Table 3.2 presents the mineral content of the representative soybean spent flake sample as measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES) as described by van de Wiel (2003). Soybean spent flakes contain significant levels of calcium (almost 1% of the total dry material weight) and potassium, with considerable amounts of phosphorous, magnesium, and sodium also detected. Note that soybean copper levels are very low (1 ppm), an observation of interest for the copper dependent oxidative enzymes described in Chapter 7.

3.2.4 Microbial levels

Soybean polysaccharides produced via the processes shown in Figures 3.1 and 3.2 contain high moisture contents (80-85%) and are able to act as ideal substrates for microbial growth due to their neutral pH and high nutrient content (Ouhida et al., 2002). For this reason, it is essential that samples are collected immediately upon production and dried as quickly as possible. Even with these precautions, bacteria present in the starting soybean white flakes will inherently be present in the spent flake waste stream. The starting white flakes and the representative spent flake sample used in this study were measured to have total plate counts of 7.8×10^3 cfu/g and 5.2×10^6 cfu/g respectively. As only a portion of the white flake solids are collected as spent flakes, a concentration of the bacteria level is observed during the initial separation

step of the process. For these reasons, it is often a necessity to include bacteriostatic agents when working with soybean spent flakes. This is particularly relevant for enzymatic reactions, which are often performed under ideal conditions for bacterial growth. Section 6.3 describes the use of bacteriostatic agents for enzymatic treatments in more detail.

Table 3.2 - Representative soybean spent flake mineral content in parts per million (ppm) as measured by ICP-AES (van de Wiel, 2003).

Mineral	Dry-basis (ppm)
Sodium	684
Potassium	7175
Calcium	9396
Phosphorus	1092
Magnesium	1204
Iron	44
Manganese	5.5
Zinc	10.7
Copper	1

3.3 Polysaccharide types

Combining the monosaccharide composition data presented in Subsection 3.2.1 with knowledge of the common polysaccharide classes presented in Section 2.1, it is possible to begin to characterize the polysaccharide classes of soybean. However, for a complete understanding and characterization, a full linkage analysis is required. This work was conducted as part of this thesis and is presented in detail in Paper 1. While the work presented in Paper 1 is the first example of a complete linkage analysis of the intact soybean polysaccharides, there have been a variety of structural studies on chemically extracted soybean cell wall components in the past. The various extraction procedures and the results of this work are further described in Section 4.1. However, the main findings and identified polysaccharides will be summarized here.

In the 1960s, G.O. Aspinall and M. Morita performed the first structural characterization work on soybean polysaccharides (Aspinall & Whyte, 1964; Morita, 1965a). In their work, the authors identified the monosaccharide composition and linkages for a variety of chemically extracted polysaccharide fractions and used this information to discern valuable structural information about the material. This work identified six principal polysaccharide classes in soybean cell walls: arabinogalactan, arabinan, pectin (galacturonan/rhamnogalacturonan), arabinoglucuronoxylan, cellulose, and xyloglucan (Aspinall, 1988). In addition, they identified that soybean hulls contain primarily cellulose, galactomannan, and xylan.

In the 1990s and 2000s, renewed interest in soybean pectin led to several structural studies of soybean polysaccharide fractions. This work was driven in part by the development of a commercialized soybean pectin product, Fuji Oil SSPS, which is described in further detail in Subsection 4.2.1. Early studies confirmed the presence of galacturonan and rhamnogalacturonan regions in the soybean pectin fraction in addition to significant amounts of netural arabinan and galactan branching associated with these regions (Yamaguchi, Ota, et al., 1996). Huisman et al. confirmed these findings. They also speculated about the presence of xylogalacturonan regions based on the observation of disaccharides of xylose and galacturonic acid following enzymatic degradation (1998; 1999). In addition, Nakamura et al. utilized the enzymatic degradation approaches detailed in Chapter 6 to propose a structure for soybean pectin consisting of galacturonan regions interspersed with rhamnogalacturonan regions that are heavily branched with galactan and arabinan sidechains (2002b). More recent studies have also confirmed these findings concerning the structure of soybean pectin (Li, Lu, et al., 2012; Mateos-Aparicio, Redondo-Cuenca, & Villanueva-Suárez, 2010). Notably absent from these previous studies are quantifications of the amounts of the polysaccharide classes in soybean. This data was determined for the first time in this thesis work and is presented in detail in Chapter 5 and Paper 1.

3.4 Applications

As described in Section 1.1, the majority of commercially produced soybean polysaccharides are currently utilized in low value applications or disposed of as waste (Li et al., 2013). Nevertheless, some portions of this material are currently used in a variety of food applications. As described in this thesis, the inherent insolubility of this material limits the potential food applications; however, promising results can be achieved through solubilization of the raw material (described in detail in Chapter 4). To illustrate this fact, it is worthwhile to note that most of the recent research on soybean polysaccharide applications has been conducted using the Fuji Oil Co. Ltd. commercialized product, soluble soybean polysaccharides (SSPS). Subsection 4.2.1 provides background information on this material and details its production process, so this information will not be repeated here. Instead, the aim of this section is to describe the possible applications for soybean polysaccharides and to highlight the significant increase in possible applications introduced through material solubilization.

3.4.1 Emulsifying and protein stabilization

Native soybean polysaccharides are water insoluble and have no inherent emulsifying or protein stabilization properties; however, solubilization of the material can impart these properties. One example of a solubilized soybean polysaccharide product is SSPS. This material has been demonstrated as both an emulsifier and emulsion stabilizer for a wide variety of applications such as flavor emulsions, powdered flavors, coffee creamers, and dressings

(Maeda & Nakamura, 2009; Nakamura, Takahashi, Yoshida, Maeda, & Corredig, 2004). This emulsifying property is present from pH 3-7 and is also pH stable. This stability indicates that the mode of action is not charge related. Instead, it may be the result of SSPS protein anchoring to the oil-water interface with hydrophilic polysaccharide chains to form a hydrated layer (Liu, Verespej, Corredig, & Alexander, 2008). For this reason, a strong connection exists between the SSPS molar mass and its emulsifying properties. This molar mass can be tailored to meet the needs of the application through changes in the extraction process (described in Subsection 4.2.1) or enzyme hydrolysis (Nakamura, Maeda, & Corredig, 2006). The performance of SSPS has been compared with other common emulsifiers (e.g. modified starch, sugar beet pectin, and gum arabic), with the results suggesting that SSPS has both functional advantages and disadvantages over these materials (Nakamura et al., 2004; Nakauma et al., 2008). In addition, SSPS has demonstrated efficacy in the formation of multi-layer emulsions (utilizing emulsifier combinations) with both sugar beet pectin (Zhao, Wei, Wei, Yuan, & Gao, 2015) and chitosan (Hou et al., 2010).

In addition to its oil-water emulsifying properties, SSPS has been shown to prevent protein coagulation and precipitation under acidic conditions (pH < 4). For example, SSPS has shown the ability to stabilize milk proteins under acidic conditions, preventing the aggregation of caseins and precipitation in applications such as drinking yogurt (Asai et al., 1994; Nakamura, Furuta, Kato, Maeda, & Nagamatsu, 2003; Nobuhara et al., 2014). This same stabilizing behavior has also been demonstrated for soy protein isolate emulsions at low pH (Roudsari, Nakamura, Smith, & Corredig, 2006). For this application, an advantage of SSPS over other commonly used materials such as carboxymethylcellulose (CMC), propylene glycol alginate, and high methoxyl pectin, is its ability to maintain a low viscosity at low pH. In addition, a unique aspect of SSPS for this application is the presence of large neutral sidechains on the anionic polysaccharide backbone, resulting in a thick coating layer around the proteins (Maeda & Nakamura, 2009). Recent results have suggested that the stability of acidified milk drinks can be improved with the combination of SSPS and CMC (Ntazinda, Cheserek, Sheng, Meng, & Lu, 2014). This protein stabilization property is believed to be the result of SSPS anionic polysaccharide interactions with the cationic protein surfaces, coating the particles and preventing aggregation through electrostatic repulsion (Maeda & Nakamura, 2009).

3.4.2 Health and nutrition

Soybean polysaccharides also have the potential for applications in the areas of health and nutrition. In their native insoluble form, their primary application is in fiber fortification of dry-blended beverages and as fiber additives to baked goods and extruded snacks. The DuPont™ FIBRIM® product line is a commercialized soybean polysaccharide product suitable for use in clinical nutri-

tion applications (FIBRIM® 2000), weight management applications (FIBRIM® 1020), bakery applications (FIBRIM® 1270), and extrusion applications (FIBRIM® 1280). In addition, DuPont™ CENERGY® FMS is a specialized soybean polysaccharide product which can be used for fiber addition and increased water retention in ground meat applications. As shown in Table 3.3 for both soybean spent flakes and okara, soybean polysaccharides contain high levels of dietary fiber. The higher carbohydrate levels in the spent flakes (Table 3.1) lead to a higher dietary fiber content in comparison with okara. In addition, SSPS has been reported to contain 66.2% dietary fiber, providing a water-soluble dietary fiber source for food applications (Maeda & Nakamura, 2009).

Table 3.3 - Representative dietary fiber composition of commercial soybean spent flakes (n=9) and okara (Mateos-Aparicio, Redondo-Cuenca, Villanueva-Suárez, Zapata-Revilla, & Tenorio-Sanz, 2010) on a dry basis. Dietary fiber measured by the enzymatic-gravimetric AOAC method 991.43 (AOAC, 1995) where TDF is Total Dietary Fiber, IDF is Insoluble Dietary Fiber, and SDF is Soluble Dietary Fiber.

Material	TDF (%)	IDF (%)	SDF (%)
Soybean spent flakes	84.0 ± 2.7	78.0 ± 2.6	5.9 ± 1.1
Okara	54.3 ± 2.3	50.1 ± 2.9	4.2 ± 1.8

Dietary fiber consumption has been shown to have significant effects on all aspects of gut physiology including changes to gut transit times, alterations to gut microflora diversity, and modifications to the immune responses of gut mucosa (Brownlee, 2011). In addition, dietary fiber intake has demonstrated diverse health benefits affecting cardiovascular disease, diabetes, cancer, and weight management ("Position of the American Dietetic Association: Health implications of dietary fiber," 2008). Despite these numerous benefits, the American Dietetic Association reports that the average American consumes only 15 g/day of dietary fiber, well short of the recommended 25-30 g/day (2008). Increased addition of dietary fiber to foods can help to reverse this trend. In particular, solubilization of soybean polysaccharides significantly increases the number of available applications for dietary fiber addition. For example, SSPS can be utilized for fiber fortification of well-established dairy foods in beverages, ice creams, and puddings with minimal deleterious sensory effects (Chen, Duizer, Corredig, & Goff, 2010). Native soybean polysaccharides have also demonstrated prebiotic efficacy, which can be enhanced through high hydrostatic pressure enzymatic treatment to increase the soluble dietary fiber content (Pérez-López, Cela, Costabile, Mateos-Aparicio, & Rupérez, 2016). In addition, water-soluble soybean polysaccharides have also shown prebiotic potential by selectively stimulating the growth of bifidobacteria (Kemperman & Mellema, 2011).

Outside of these effects, soybean polysaccharides have also shown additional health benefits. The effects of soybean polysaccharides on blood sugar and blood lipid metabolism in diabetic mice have been studied by Xu et al. In their

study, blood sugar and total serum cholesterol were markedly reduced, and high-density lipid cholesterol was increased significantly. The results of this work demonstrated that soybean polysaccharides (okara) can dramatically reduce plasma sugar and lipid levels, indicating potential applications in diabetes prevention and treatment (Xu, Tan, & Li, 2001). In addition, studies have shown that the inclusion of soybean polysaccharides in high-fat diets can result in a lowering of plasma triglycerides and total cholesterol levels, as well as an increase in fecal extraction of lipids, triglycerides, and cholesterol (Villanueva, Yokoyama, Hong, Barttley, & Rupérez, 2011; Wang & Li, 1996). These results indicate possible applications for soybean polysaccharides in hyperlipidemia prevention. Soybean polysaccharides have also demonstrated potential utility for obesity prevention. Rats fed a high-fat diet supplemented with okara showed a suppression of weight gain and a prevention of increased epididymal white adipose tissue (Préstamo, Rupérez, Espinosa-Martos, Villanueva, & Lasunción, 2007). In addition, okara was shown to induce the down-regulation of the fatty acid synthetase gene in the liver (Matsumoto, Watanabe, & Yokoyama, 2007). Finally, soybean polysaccharides have also demonstrated antioxidant activity. While this has been primarily attributed to residual protein in the material (Yokomizo, Takenaka, & Takenaka, 2002; Zhu, Fan, Cheng, & Li, 2008), alkali soluble fractions have also shown this effect and indicate that soybean pectins may also play a role in this function (Mateos-Aparicio, Mateos-Peinado, Jiménez-Escrig, & Rupérez, 2010).

3.4.3 Texture and gelation

The soluble soybean polysaccharides extracted in the SSPS process have also shown utility in a variety of functional food applications due to their unique physical properties. For example, SSPS shows high adhesive strength, allowing it to function as a binder in diverse food applications. In addition, SSPS demonstrates film forming capability and can be used to form a colorless and water-soluble edible film for food applications (Furuta, Tobe, Kiwata, & Maeda, 1999; Maeda & Nakamura, 2009). SSPS can also be utilized to soften the texture of many food products such as breads, meats, cakes, and cream sauces (Maeda & Nakamura, 2009). Lastly, SSPS has been utilized for its anti-sticking effect on cooked rice and noodles. The application of SSPS to cooked rice and noodles prevents these materials from sticking for several hours after cooking. In addition, SSPS can be added during the cooking of rice and noodles to prevent any hardening effects during cold storage of the final product. The mechanism for these properties appears to be driven by the galacturonic acid backbone of the SSPS forming a coating layer on the surface of the rice or noodles (Furuta et al., 2003; Ishihara et al., 2010; Maeda & Nakamura, 2009).

3.4.4 Others

Outside of the primary applications described in Subsections 3.4.1-3.4.3, soybean polysaccharides have also been used for a variety of diverse applica-

tions. As previously described, soybean polysaccharides which are not disposed of as waste or burned are typically used in lower value applications such as animal feed or as mushroom growth media (Kasai, Murata, Inui, Sakamoto, & Kahn, 2004; Li et al., 2013; Wong, Tabg, & Kwok, 1996). For animal feed, the polysaccharides are often included as a replacement for the more expensive soybean meal in the diets of cattle, pigs, goats, fish, and chickens (Li, Qiao, & Lu, 2011). In addition, soybean polysaccharides can serve as an excellent substrate for microbial fermentation, and a variety of fermented okara products have been developed for both food and non-food applications (Li et al., 2011). In addition, SSPS can be used for the microencapsulation of materials to prevent unwanted oxidation and improve storage stability (Fang et al., 2003; Hojjati, Razavi, Rezaei, & Gilani, 2011), and SSPS has shown utility in foam stabilization of food products such as meringues (Maeda & Nakamura, 2009).

Non-food applications have also been demonstrated in the absorption of organochlorine compounds in waste water treatment (Adachi, Hamamoto, & Okano, 2005), as an environmentally friendly wood preservative (Ahn et al., 2010), and as an additive to rubber composites (Jong, 2007). Soybean polysaccharides have also been used to generate edible and/or highly biodegradable packaging (Li et al., 2007; Wen & Liu, 2007; Zhang, Liu, & Xu, 2008). Also, alkali extracted soybean polysaccharides can be used to form nanofibers, with potential applications in nanoencapsulation formation for drug delivery (Fung, Yuen, & Liong, 2010).

3.5 Challenges

Sections 3.2 and 3.3 have demonstrated that the cell walls of soybean polysaccharides are complex and heterogeneous. This complexity creates unique analytical challenges when working with this material. Often, additional work must be conducted to adapt the methods described in Section 2.2 to the analysis of soybean polysaccharides.

As described in Subsection 2.2.1, monosaccharide analysis requires that the polysaccharides are fully hydrolyzed to their monosaccharide constituents prior to analysis. However, soybean polysaccharides contain several polysaccharide classes of varying recalcitrance, often requiring differing hydrolysis conditions. If the conditions employed are too mild, insufficient hydrolysis of the cellulosic polymers will underrepresent the xylose and glucose levels of the material. If the conditions employed are too severe, degradation of more labile monosaccharides such as fucose can occur. For this reason, it is essential to develop a hydrolysis method that addresses this challenge, either by utilizing spiked internal standards as a measure of degradation, or performing two different hydrolyses to fully capture the complete monosaccharide profile. For the work conducted in this thesis, a hydrolysis method was designed specifically for soybean polysaccharides based on recovery of polysaccharide and monosaccharide standards (full method presented in Paper 1). This

method gives excellent recovery of both labile and non-labile soybean monosaccharides through the use of a concentrated acidic pretreatment at low temperature, followed by a weakly acidic, high temperature hydrolysis in an autoclave. Following the successful hydrolysis of the material, there are still challenges associated with the efficient separation and quantification of the eight resulting monosaccharides. As a part of this project, a customized HPAEC-PAD method was created to give baseline separation of these monosaccharide constituents for accurate quantification (details presented in Paper 1).

Early work for this project attempted to utilize the NMR techniques described in Subsection 2.2.3 for the structural characterization of soybean polysaccharides. However, the insolubility and heterogeneity of the material gave convoluted results that did not provide sufficient information. In order to employ these NMR techniques for a complex cell wall polysaccharide substrate like that found in soybean, it is required, in most cases, that the sample first undergo a series of extractions, separations, and purifications. If purified and homogeneous samples can be obtained, NMR analysis is an excellent tool for carbohydrate structural characterization. Extraction and solubilization approaches are discussed in greater detail in Chapter 4.

4. Solubilization of soybean polysaccharides

The insolubility of soybean polysaccharides has limited their use as a food additive or functional food ingredient. In addition, their lack of solubility has limited their structural characterization, as the vast majority of analytical techniques require soluble material for analysis. For these reasons, techniques for the solubilization and purification of soybean polysaccharides are of paramount interest (Canut, Albenne, & Jamet, 2017). This chapter will detail the traditional chemical extraction techniques that can be employed to extract various polysaccharide types from cell wall polysaccharides. In addition, specific processes for the solubilization of soybean polysaccharides will be described in further detail. As enzymatic treatment is a significant tool in polysaccharide degradation and solubilization, the entirety of Chapters 6 and 7 are dedicated to this topic and their application has been omitted from this chapter.

4.1 Extraction of cell wall components

For the structural characterization of cell wall polysaccharides, it is often necessary to obtain homogenous carbohydrate fractions for analysis. However, this is not a simple task for complex polysaccharide systems, such as those found in soybean, which often exist in a heterogeneous form (Albersheim, Darvill, Roberts, Sederoff, & Staehelin, 2010). In order to address this problem, a generalized approach can be applied to sequentially extract various cell wall polysaccharide classes by their chemical solubility. As a first step, any starch or protein present in the sample is removed. The protein removal is typically performed to purify the carbohydrate fraction, and the starch removal is typically done because the presence of starch can complicate further structural characterization as described in Subsection 2.2.2. Next, the cell walls are typically treated with chelating agents that are capable of solubilizing the pectin polysaccharides. Following this step, extractions with solutions containing increasing alkaline strength can be used to extract hemicelluloses. Finally, the remaining residue consists of the cellulosic polysaccharides (Wrolstad et al., 2005). These treatments are described in more detail below, and examples of their application to the solubilization of soybean polysaccharides are given.

4.1.1 Starch and protein removal

As described previously, it is typically desirable to remove any residual starch from a cell wall polysaccharide sample prior to compositional and structural analysis. As starch contains 1,4-linked glucose residues, it is difficult to discern the proportion of sample glucose contributed by starch or cellulose without this step. While starch can be present in germinating soybean cotyledons, typically only trace amounts are detected in mature soybean seeds (Wilson et al., 1978). A standard approach for starch removal is to utilize porcine pancreatic

α-amylase (EC 3.2.1.1) for enzymatic degradation. This enzyme is highly specific for the α-1,4-linked glucose residues in starch and will leave other glucan polysaccharides in the sample untouched. This is the same approach employed in the linkage analysis described in Subsection 2.2.2. Huisman et al. and Ouhida et al. found that two 19 h incubations with α-amylase were sufficient for starch removal from soybean polysaccharides and utilized this procedure prior to the extractions described below (Huisman et al., 1998; Ouhida et al., 2002). Another approach is to utilize 90% dimethyl sulfoxide to solubilize starch for removal by rinsing (Aspinall, 1988).

As described in Chapter 3, the primary soybean polysaccharide source utilized in this thesis work was soybean spent flakes. This product results from the protein extraction of dehulled and defatted soybean white flakes. However, for studies characterizing soybean polysaccharides from other sources, it is important to first remove residual protein from the sample in order to purify the carbohydrate components. This can be accomplished through extraction with a 1.5% (w/v) sodium dodecyl sulfate (SDS) solution containing 1,4-dithiothreitol (Huisman et al., 1998; Ouhida et al., 2002). Other approaches involve the use of deoxycholate in phenol-acetic acid-water or enzymatic digestion with protease (Aspinall, 1988; Selvendran, 1975).

4.1.2 Pectin extraction

Extraction of pectin polysaccharides typically involves the use of chelating agents to disrupt cross-linkages holding the polysaccharides together. While the wide variety of functional groups in pectin creates ample opportunities for many types of cross-linkages to occur, it has been a long-held belief that calcium cross-linkages contribute to the insolubility of pectin. Through the use of chelating agents, these cross-links can be disrupted and a portion of the pectin polysaccharides can be extracted into the soluble fraction (Fry, 1986).

Early approaches by Aspinall et al. utilized ammonium oxalate and ethylenediaminetetraacetic acid (EDTA) for this purpose (1967). These fractions were used to establish the presence of both acidic and arabinogalactan polysaccharides in the solubilized material, which subsequently led to the conclusion that the pectin fraction of soybean contains a significant amount of neutral sugar branching. More recently, Li et al. used this same extraction to obtain a 7.7% yield from the starting crude okara polysaccharide (Li, Lu, et al., 2012). This fraction contained predominantly galacturonic acid, galactose, and arabinose, confirming the findings of Aspinall. In addition to this treatment, Yamaguchi et al. explored the use of hexametaphosphate for the extraction of soybean pectin. In their work, they confirmed the previous findings of Aspinall et al. and found that the extracted pectin fraction contained both galacturonans and rhamnogalacturonans which were substituted by arabinans and galactans (Yamaguchi, Kojima, Muramoto, Ota, & Hatanaka, 1996; Yamaguchi, Ota, et al., 1996).

Outside of these extraction methods, extracts utilizing 1,2-cyclohexylenedinitrilotetraacetic acid (CDTA) and oxalate have also been tested on soybean polysaccharides. In a similar fashion to the EDTA and hexametaphosphate extracts, this fraction contains primarily galactose, arabinose, and galacturonic acid. Enzymatic removal of the galactose and arabinose residues, methods for which are discussed in more detail in Chapter 6, resulted in a fraction that was classified as xylogalacturonan and rhamnogalacturonan (Huisman et al., 2001; Ouhida et al., 2002). Solid-state NMR analysis, namely cross polarization magic angle spinning (CP-MAS), has also been conducted on this CDTA soluble fraction to estimate the degree of esterification and enzymatic degradation properties (Fransen, van Laar, Kamerling, & Vliegenthart, 2000). Mateos-Aparicio et al. also investigated this extraction process and reported an overall extraction yield of 7.2%. They obtained similar results to those of previous researchers, showing a fraction containing slightly methylated rhamnogalacturonan heavily substituted with arabinans, galactans, and arabinogalactans (Mateos-Aparicio, Redondo-Cuenca, & Villanueva-Suárez, 2010). However, their reported extraction yield was significantly lower than the 38% reported by Huisman et al. for their CDTA extraction (Huisman et al., 1998).

An interesting approach that can be used for analysis of the extracted pectin polysaccharides is treatment with lithium in 1,2-diaminoethane (Lau, McNeil, Darvill, & Albersheim, 1987). This technique results in the degradation of uronic acid residues, leaving any neutral residues and their linkages to other neutral residues intact. Using this technique, it is possible to evaluate the intact neutral side chains on pectin polysaccharides. Fransen et al. applied this method for the characterization of the chelating agent soluble extract of soybean polysaccharides (Fransen, Haseley, et al., 2000). They confirmed the presence of arabinogalactan sidechains in the pectin polysaccharides which consist of β-1,4-linked galactose residues glycosidically linked to the 4-position of 1,2-linked backbone rhamnose residues in rhamnogalacturonan I and substituted with branched arabinan chains.

4.1.3 Hemicellulose extraction

One approach for the extraction of hemicellulosic polysaccharides from the cell wall matrix is alkaline extraction, which is typically performed following the pectin polysaccharide extraction described in Subsection 4.1.2. Alkali treatment is capable of disrupting the cell wall interactions by cleaving hydrogen bonds, covalent bonds, and ester linkages in the polysaccharide matrix (Sun, Wen, Ma, & Sun, 2013). By altering the concentration of the alkali treatment, it is possible to sequentially extract different hemicellulosic polysaccharides, with higher concentrations of alkali releasing the more tightly imbedded polysaccharides. These extractions are typically performed with sodium hydroxide (NaOH), potassium hydroxide (KOH), or a combination of the two.

Huisman et al. performed an alkaline sequential extraction on an already CDTA extracted soybean polysaccharide sample (1998), adopting the extraction conditions proposed by Redgwell and Selvendran (1986). This involved the use of alkali concentrations of 0.05 M NaOH, 1 M KOH, and 4 M KOH, resulting in extraction yields of 7%, 16%, and 7% respectively. The 0.05 M NaOH extracted fraction - the so called dilute alkali soluble solids (DASS) fraction - consisted primarily of galactose, galacturonic acid, and arabinose. The 1 M KOH fraction was primarily galactose, arabinose, and galacturonic acid, while the 4 M KOH fraction contained primarily xylose and glucose. The residual insoluble fraction following all extractions was primarily glucose. Ouhida et al. performed the same extraction and obtained similar results, although the 4 M KOH extract contained less glucose and xylose (2002). Finally, Li et al. recently performed a similar sequential extraction on an EDTA extracted soybean polysaccharide sample, using only NaOH (Li, Lu, et al., 2012). They obtained similar results to Huisman et al. and Ouhida et al.; however, the 4 M alkaline extraction fraction was primarily galactose and arabinose and did not contain the higher glucose levels previously observed with KOH extractions. In addition, the DASS fraction (0.05 M NaOH) from this extraction was further investigated with solid state CP-MAS NMR and showed a lower degree of esterification when compared with the pectin fractions extracted with chelating agents.

One challenge with this approach is that these sequential extraction steps rarely result in non-overlapping fractions for legumes, as they might for woods or cereals (Aspinall, 1988). For this reason, it is important to use caution when interpreting compositional data from these extractions, bearing in mind that the fractions are most likely not purified fractions of a single polysaccharide class. However, additional purifications can be accomplished using chromatographic and/or enzymatic approaches.

4.2 Specific processes

While the extractions described in Section 4.1 are useful generalized approaches for the separation and purification of soybean polysaccharides, this section will focus in more detail on specific processes that have been employed to generate a solubilized soybean polysaccharide fraction. The peroxide solubilization process (Subsection 4.2.2) is described in significantly greater detail in Paper 1; however, it is introduced and briefly described here for completeness.

4.2.1 Soluble soybean polysaccharides (SSPS)

In 1993, Fuji Oil Co. Ltd. in Japan introduced a soybean polysaccharide product to the market under the brand name SOYAFIBE-S. This material, also referred to as soluble soybean polysaccharides (SSPS), consists of water-soluble polysaccharides which have been extracted from soybean okara (Maeda et al., 1992). This product is marketed for its ability to enhance protein stability

in acidic beverages and desserts, as well as its ability to maintain the quality of cooked noodles and rice. Figure 4.1 depicts a generalized SSPS production method outlined by Cui et al. (2006), where the okara is first obtained from soy milk or tofu production as described in Section 3.1. This process represents a further evolution of the hot water extraction process first proposed by Morita in the 1960s (Morita, 1965a).

The key process step involves the acidic extraction of polysaccharides under pressure in an autoclave, where the target polysaccharides are liberated from the remaining cell wall polysaccharides (unextracted solids). In addition, the concentration and drying step is also important for the removal of unwanted salts from pH adjustments during the production process. A variety of process operations are feasible for this task, and published information on the production process does not explicitly specify the preferred method. More recent patents and publications on the SSPS process highlight additional operations that can be performed to tailor the level of esterification in the resulting polysaccharides. These include de-methylesterification with alkaline treatment (pH 9-13) at 40 °C and acetic acid esterification at neutral or alkaline pH (Yoshida, Sato, & Nakamura, 2014). It should also be noted that the process described in Figure 4.1 can also be performed with soybean spent flakes resulting from the soy protein isolate production process. In fact, the lower protein content and higher overall purity of the polysaccharides in this material make it more ideally suited for the SSPS extraction process (Yoshida et al., 2014).

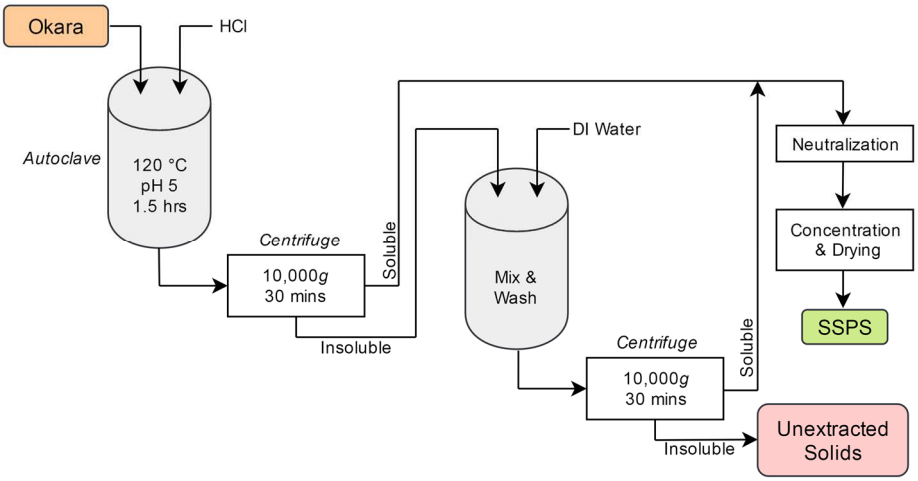

Figure 4.1 - Generalized production process for SSPS (Cui et al., 2006). The starting okara, colored orange, is produced as described in Section 3.1 and the final product is colored green.

There have been a variety of reports on the composition and structure of the polysaccharides resulting from the SSPS extraction process. In general, SSPS contains galactose (46.1%), arabinose (22.6%), galacturonic acid (18.2%), rhamnose (5.0%), xylose (3.7%), fucose (3.2%), and glucose (1.2%).

The majority of these monosaccharides are contained within long-chain rhamnogalacturonan and short-chain homoglacturonan polysaccharides decorated with galactose and arabinose neutral side chain residues (Nakamura et al., 2002b). SSPS has been described to have a diverse set of material properties including a high dietary fiber content, high solubility, stable viscosity across pH and salt ranges, adhesive properties, film forming abilities, and anti-oxidative properties (Maeda & Nakamura, 2009). This range of properties has led to the development of a range of SOYAFIBE products that are used as soluble dietary fibers, protein stabilizers for acidic beverages, emulsifiers, film forming agents, foam stabilizers, dispersing agents, softeners, and anti-sticking agents. These applications are described in more detail in Section 3.4.

4.2.2 Peroxide solubilization

In the work for this thesis, a novel approach for the solubilization of soybean polysaccharides was developed. This process involves the treatment of soybean polysaccharides with hydrogen peroxide (3% w/w) at a neutral pH, and it is capable of extracting up to 70% of the soybean spent flake solids as high molar mass, water-soluble polysaccharides. The development of this process and the impact of the various parameters (peroxide concentration, reaction time, and reaction pH) on the yield and resulting polysaccharide properties are described in detail in Paper 1 and will not be repeated here. However, it is worthwhile to compare the polysaccharides obtained through this extraction with those generated in the SSPS process. In general, the peroxide solubilized polysaccharides contain galactose (44.6%), arabinose (27.4%), galacturonic acid (10.9%), xylose (5.9%), glucose (4.5%), rhamnose (3.5%), and fucose (3.1%). These results are not too dissimilar to those presented for SSPS in Subsection 4.2.1, but a reduction in galacturonic acid and increase in xylose and glucose suggests a greater proportion of solubilized xyloglucan. The constituent polysaccharides reported from the linkage analysis conducted in Paper 1 confirm that the two extractions result in similar polysaccharides, with type I arabinogalactan (37.1%), (glucurono)arabinoxylan (18.3%), rhamnogalacturonan (11.1%), and arabinan (9.6%) being the primary polysaccharide classes identified in the peroxide solubilized fraction (Pierce et al., 2016).

4.3 Summary of chemical solubilization approaches

As discussed above in Sections 4.1 and 4.2, there have been a variety of approaches for the chemical solubilization of soybean polysaccharides. Table 4.1 provides a summary of this work, in an effort to condense the presented information into a format which can be easily referenced. Note that the majority of the approaches have centered around the solubilization and removal of protein with SDS/DTT, followed by the chelating agent assisted extraction of pectin with CDTA/oxalate, and finally sequential alkaline extractions of hemicellulose using sodium hydroxide and/or potassium hydroxide. In addition, there have been a variety of studies utilizing the SSPS solubilization process detailed in Subsection 4.2.1.

Table 4.1 – Summary of chemical solubilization approaches on soybean polysaccharides. Studies are identified with their reference ID, with a key provided in chronological order at the bottom of the table.

Solubilization Treatment	Reference ID(s)	Purpose
Sodium dodecyl sulfate (SDS) solution containing 1,4-dithiothreitol (DTT)	J, K, L, M, O, S, U, V	Remove protein
Deoxycholate in phenol-acetic acid-water	F, G	
Dimethyl sulfoxide (DMSO)	G	Solubilize starch
Sodium hypochlorite	X	Remove lignin
Ammonium oxalate	C, D, V	Extract pectin
Ethylenediaminetetraacetic acid (EDTA)	C, D, V	
1,2-Cyclohexylenedinitrilotetraacetic acid (CDTA) and oxalate	J, K, L, M, O, S, U	
Hexametaphosphate	I	
Sodium Hydroxide	A, B, C, D, E, J, K, M, S, U, V, X	Extract hemicellulose
Potassium Hydroxide	C, J, K, S, U	
Lithium in 1,2-diaminoethane	L	Degrade acidic monomers
SSPS Treatment (see 4.2.1)	H, N, P, Q, R, T, W	Solubilize pectin
Peroxide Treatment (see 4.2.2; Paper 1)	Y, Z	Solubilize non-cellulosic polysaccharides

Reference ID Key: **A**: (Morita, 1965a); **B**: (Morita, 1965b); **C**: (Aspinall, Hunt, & Morrison, 1966); **D**: (Aspinall et al., 1967); **E**: (Morita, Okuhara, Kikuchi, & Sakurai, 1967); **F**: (Aspinall & Cottrell, 1971); **G**: (Aspinall, 1988); **H**: (Maeda et al., 1992); **I**: (Yamaguchi, Ota, et al., 1996); **J**: (Huisman et al., 1998); **K**: (Huisman et al., 1999); **L**: (Fransen, Haseley, et al., 2000); **M**: (Fransen, van Laar, et al., 2000); **N**: (Nakamura, Furuta, Maeda, Nagamatsu, & Yoshimoto, 2000); **O**: (Huisman et al., 2001); **P**: (Nakamura, Furuta, Maeda, Nagamatsu, & Yoshimoto, 2001); **Q**: (Nakamura et al., 2002a); **R**: (Nakamura et al., 2002b); **S**: (Ouhida et al., 2002); **T**: (Wang, Huang, Nakamura, Burchard, & Hallett, 2005); **U**: (Mateos-Aparicio, Redondo-Cuenca, & Villanueva-Suárez, 2010); **V**: (Li, Lu, et al., 2012); **W**: (Yoshida et al., 2014); **X**: (Ratnadewi, Handayani, Oktavianawati, Santoso, & Puspaningsih, 2016); **Y**: (Pierce et al., 2016); **Z**: (Paullin, Nambiar, Tran, & Pierce, 2016).

5. Paper 1: Formation of water-soluble soybean polysaccharides from spent flakes by hydrogen peroxide treatment

Pierce, B. C.; Wichmann, J.; Tran, T. H.; Cheetamun, R.; Bacic, A.; & Meyer, A. S. 2016. Carbohydrate Polymers, 144, 504-513.

This chapter contains a discussion of Paper 1 and its role in the work presented in this thesis. The full text of this paper can be found in the Publications section at the end of this thesis.

Brief summary
Paper 1 describes a novel chemical process for the solubilization of soybean spent flake polysaccharides using hydrogen peroxide. In addition, results of a two-year survey on the composition of soybean spent flakes are presented. Finally, GC-MS linkage analysis results are presented for both the native spent flake material and the products of the peroxide solubilization process, providing quantitative compositional information on the polysaccharide classes present in these materials.

Research goals
As presented in Chapter 3, the production process of soy protein isolate results in the generation of soybean spent flakes, a by-product enriched in the cell wall polysaccharides of soybean. One goal of the research presented in this paper was to further understand the composition of this material and its consistency across different processes and crop years. At the outset of this project, it was hypothesized that the carbohydrate composition of this material would remain highly consistent across an extended sampling period, and a primary goal of this work was to test this hypothesis.

While polysaccharide characterization for soybeans has been previously described, these studies have relied exclusively on chemical solubilization of extracts for sequential analysis (further described in Section 3.3 of Chapter 3 and in Chapter 4). A goal of this research was to conduct a holistic linkage analysis of the entire native spent flake material, in an effort to capture additional linkage information which may be lost during chemical extraction. In particular, efforts were made to categorize the constituent polysaccharides into the classes described in Section 2.1. Moreover, a primary goal was to quantify the levels of these polysaccharide classes, thereby addressing a shortcoming of previous research on this material. This information was necessary to address a central hypothesis of this work, namely that detailed knowledge of the polysaccharide composition will unlock new opportunities for further modification of this material.

The research in Paper 1 also examines a novel chemical solubilization process for soybean polysaccharides utilizing hydrogen peroxide. The results of this solubilization were used to test the project hypotheses that chemical treatment could not only result in polysaccharide solubilization, but also that this treatment could be used to selectively tailor the properties of the resulting products. A primary goal for the development of this solubilization process was to further understand the connection between the polysaccharide composition of the material and the fundamental lack of water solubility exhibited by the spent flakes. An underlying hypothesis of this work was that the structural make-up of the polysaccharides dictates this insolubility, and efforts to test this hypothesis were conducted through compositional analysis of the water-soluble polysaccharides generated by this process.

Main results

Forty-four commercial soybean spent flake samples, produced from both genetically modified (GM) and identity preserved (IP) soybean strains, were collected over the course of 22 months. The results show remarkable consistency in the composition of this material, with no statistically significant differences observed between the GM and IP produced materials. As shown in Figure 2 of Paper 1, the sugar composition measured by acid hydrolysis and HPAEC-PAD was also extremely consistent. This consistency allowed a representative spent flake sample to be chosen for the subsequent analyses performed in this project. In addition to the consistency in carbohydrate composition, consistency in protein, fat, and mineral content was observed. These levels are extremely vulnerable to variations in the production process, for example, higher observed mineral levels due to the addition of excess acid or base for pH adjustments. However, these results highlight the consistency in the soy protein isolate process and are a reflection of the relatively early point at which the soybean spent flakes are separated in this process.

The complete GC-MS linkage analysis of the representative spent flake sample is given in Table 4 of Paper 1 and is reproduced here in Table 5.1 for ease of reference. The most abundant linkage types observed were 1,4-linked galactose (26.8%), 1,4-linked glucose (24%), terminal arabinose (12.3%), 1,4-linked xylose (9.8%), 1,4-linked galacturonic acid (4.7%), and 1,2-/1,2,4-linked rhamnose (3%). These linkages illustrate the presence of several primary polysaccharide classes in the soybean spent flakes, namely galactan, cellulose, arabinogalactan, xylan, rhamnogalacturonan, and homogalacturonan. A notable finding is the high-observed level of terminal arabinose, indicating the presence of significant arabinose sidechains in arabinogalactan, xyloglucan, and xylan. This is underscored by the identification of significant levels of branch points for the backbone residues of these polysaccharides. In addition, the identification of low levels of glucuronic acid indicate the limited presence of glucuronoarabinoxylan. While this polysaccharide class was first reported in soybean by Kato and Matsuda, it had not been reported on in subsequent studies (Kato & Matsuda, 1985). This is most likely due to its low abundance;

however, these results do confirm the identification of this monosaccharide in the HPAEC-PAD sugar profile.

It should be noted that xylogalacturonan was not observed in this work; however, it has been reported as a minor constituent of soybean pectin polysaccharides by Nakamura et al. (Nakamura et al., 2002a). In the work of Nakamura et al., the xylose residues were shown to link to the *O*-3 position of the galacturonic acid backbone residues. In the research presented in Paper 1, no detectable levels of 1,3,4-linked galacturonic acid were observed in the native spent flake material; however, minor levels were observed in the solubilized fraction. Most likely, these branches are present at very minor levels and were therefore not detected in the native material.

The quantification of the polysaccharide composition for these materials is given in Table 5 of Paper 1 and is reproduced here in Table 5.2 for ease of reference. This data represents the first quantification of the polysaccharide classes in soybean and advances the understanding of this complex substrate.

Paper 1 also presents details on the development of a peroxide solubilization process for soybean spent flakes. This process was optimized to result in a >60% yield of high molar mass, water-soluble polysaccharides with minimal release of monosaccharides (an indicator of over-degradation). A significant result of this work was evidence that the random peroxide scission mechanism utilized for this treatment could be precisely controlled to specifically tailor the molar mass of the soluble products (results shown in Paper 1 Figure 3). Table 5.1 contains the results from GC-MS linkage analysis of the soluble and insoluble products resulting from this treatment, and Table 5.2 highlights significant differences in polysaccharide composition. The peroxide solubilized fraction was significantly enriched in the pectin and hemicellulosic polysaccharide classes of soybean (arabinan, arabinogalactan, rhamnogalacturonan, homogalacturonan and arabinoxylan). In contrast, the insoluble fraction was significantly enriched in cellulose. An interesting observation was also that this fraction contained the majority of the spent flake protein and mineral content.

Conclusion

The data presented in Paper 1 corroborate Hypothesis (a) from Section 1.2, specifically that the carbohydrate composition of soybean spent flakes is highly consistent. This hypothesis was a fundamental basis for this thesis work, owing to the fact that the compositional and structural analysis of this material is only of significant interest if the results can be applied to the substrate in a generalized fashion. This observation allowed for a representative soybean spent flake sample to be selected for further investigation with chemical and enzymatic solubilization approaches. One such solubilization approach was the hydrogen peroxide solubilization process presented in Paper 1. The results from this research confirm Hypotheses (3) and (b) from Section

1.2 by demonstrating the ability of chemical treatment to both solubilize a significant portion of the recalcitrant soybean polysaccharides and to tailor the properties of the resulting soluble polysaccharides.

Table 5.1 - Linkage (molar %) analyses of soybean spent flakes and H_2O_2 solubilization products (duplicate analysis). Abbreviations: (f) = furanose; (p) = pyranose; AG = arabinogalactan; HG = homogalacturonan; HM = heteromannan; HX = heteroxylan; RG = rhamnogalacturonan; XG = xyloglucan.

		Spent Flake	**H_2O_2 Soluble**	**H_2O_2 Insoluble**	
Mono.	**Linkage**	**Mol%**	**Mol%**	**Mol%**	**Associated polysaccharide**
Ara(f)	Terminal	12.3	15.3	11.0	Type I/II AG, HX, XG, arabinan
	2-	0.4	0.1	0.5	Type I/II AG, HX, RGII
	3-	0.2	0.3	0.4	HX
	5-	2.1	2.5	2.5	Arabinan, Type II AG, HX
	3,5-	3.0	3.5	2.5	Arabinan
Ara(p)	Terminal	1.7	1.2	2.8	Type II AG
Gal(p)	Terminal	2.0	4.3	3.3	XG, Type II AG, HX, RGI, RGII
	4-	26.8	36.6	20.7	Type I AG/RGI
	3-	1.7	0.7	1.7	Type II AG
	6-	0.1	0.2	0.3	Type II AG, RGI, RGII
	3,4-	0.5	0.3	0.4	Type I AG
	2,4-	0.3	0.2	0.3	Type I AG
	3,4,6-	0.1	0.0	0.0	Type II AG
	2,3,4-	0.1	0.0	0.0	Type I AG
Glc(p)	Terminal	0.4	0.1	0.6	Glucan classes
	4-	24.0	1.7	27.0	Cellulose, XG, HM, starch
	6-	0.2	0.1	0.2	-
	3,4-	0.3	0.9	0.4	-
	2,4-	0.2	0.0	0.2	-
	4,6-	0.5	0.4	0.4	XG, starch
	3,4,6-	0.1	0.0	0.0	-
Fuc(p)	Terminal	1.4	0.7	3.1	XG

Xyl(p)	2-	1.1	2.2	1.5	XG, RGII
	4-	9.8	12.4	8.4	HX
	2,4-	2.0	2.8	1.3	HX
	3,4-	0.2	0.0	0.2	HX
Rha(p)	Terminal	0.0	0.1	0.0	Type II AG, RGII
	2-	0.9	1.4	1.5	RGI
	2,4-	2.1	4.1	1.8	RGI
Man(p)	Terminal	0.2	0.2	0.0	-
	4,6-	0.0	0.0	2.4	HM
GalA(p)	Terminal	0.2	0.5	0.6	HG, RGI, RGII
	4-	4.7	6.5	3.7	HG, RGI
	3,4-	0.0	0.8	0.0	RGI
GlcA(p)	Terminal	0.1	0.0	0.3	HX, Type II AG, RGII

In addition to these findings, the GC-MS linkage analysis results presented in Paper 1 allowed for the structural classification of soybean polysaccharides. This information was of particular importance to this project, as a central hypothesis of this work (Hypothesis (2) from Section 1.2) was that this compositional information could be utilized to develop modification approaches for this substrate. While examinations of the product functionality of the peroxide solubilized polysaccharide fraction were outside the scope of this work, the polysaccharide composition, molar mass, and water solubility all suggest that this material could be utilized in food applications such as fiber fortification, beverage stabilization, emulsification, and dispersion (Williams & Phillips, 2009). Of particular importance were the linkage analysis results of the soluble and insoluble fractions following peroxide treatment. The ready solubilization of the pectin and hemicellulose fractions under mild peroxide treatment, and the significant enrichment of cellulose in the insoluble fraction, strongly suggest a connection between the inherent water insolubility of this material and the cellulosic polysaccharides. This finding is further confirmed by the similar results observed for the chemical extractions and solubilization approaches discussed in Chapter 4. These results help to validate Hypothesis (1) from Section 1.2, that the water insolubility of soybean polysaccharides is linked to the structural make-up of the polysaccharides.

The findings of Paper 1, specifically the polysaccharide composition of soybean spent flakes and the apparent connection between the material solubility and the cellulose content, were used to develop enzymatic approaches for the degradation of this material in the subsequent thesis work. The following chapters discuss approaches for the enzymatic treatment of soybean polysaccharides. In particular, Chapter 7, Paper 2, and Paper 3 present the results for treatment of soybean spent flakes with lytic polysaccharide monooxygenases

(LPMOs), a class of enzymes capable of oxidative degradation of substrates containing recalcitrant cellulose regions.

Table 5.2 - Calculation of polysaccharide composition (mol%) of the soybean spent flakes and H_2O_2 solubilization products, based on the data from Table 5.1 and assignment criteria from Pettolino et al. (Pettolino et al., 2012). Table is reproduced from Table 5 of Paper 1 (Pierce et al., 2016). Abbreviations: AG = arabinogalactan; HG = homogalacturonan; HM = heteromannan; XG = xyloglucan.

Polysaccharide	Soybean Spent Flake	H_2O_2 Soluble	H_2O_2 Insoluble
Arabinan	8.1	9.6	7.5
Type I AG	27.8	37.1	21.6
Type II AG	2.0	0.9	2.0
XG	2.7	3.4	2.5
Rhamnogalacturonan I/II	6.2	11.1	6.7
HG	1.6	2.2	0.3
(Glucurono)arabinoxylan	14.4	18.3	11.9
HM	0.0	0.0	4.7
Cellulose	23.5	1.3	26.7
Others (Unassigned)	13.7	16.1	16.1

6. Enzymatic treatment of soybean polysaccharides

The linkage analysis results from Paper 1 and presented in Tables 5.1 and 5.2 give a description of the polysaccharide classes in soybean. For the native soybean spent flakes, the primary classes of polysaccharides identified are: arabinogalactan (29.8%), cellulose (23.5%), arabinoxylan (14.4%), arabinan (8.1%), rhamnogalacturonan/homoglacturonan (7.8%), and xyloglucan (2.7%). Note that this classification does leave 13.7% of the observed linkages uncategorized. Nevertheless, this analysis provides a blueprint of soybean polysaccharide composition and can be used to specifically tailor the modification and degradation of this material. Unlike the chemical solubilization approaches described in Chapter 4 and in Paper 1, this chapter will focus on the enzymatic degradation of soybean polysaccharides. The advantage of enzymatic degradation over the chemical approaches previously described is the ability to specifically target individual monosaccharide components and linkage types. The aim of this chapter is to present the enzyme classes that are applicable for soybean polysaccharide degradation, provide examples of how these enzymes have been used to this effect, and discuss other considerations for enzymatic treatment of this substrate.

6.1 Relevant enzyme classes

The sections below describe the various enzymes that are applicable for the degradation of the primary polysaccharide classes in soybean. Note that the polysaccharide classes have been listed in order of abundance in soybean spent flakes. For reference, Chapter 2 provides an overview of the composition and structure of these polysaccharide classes.

6.1.1 Arabinogalactan and arabinan degradation

Both arabinan and type I/II arabinogalactan are found in high abundance in soybean polysaccharides. The linkage analysis results from Table 5.2 show that soybean spent flakes contain 27.8% of type I arabinogalactan, 8.1% of arabinan, and 2.0% of type II arabinogalactan. The primary cleavages required for the degradation of these polysaccharide classes involve the breaking of β-1,4-galactose and α-1,5-arabinose linkages. For this purpose, there are several key enzymes to consider.

endo-β-1,4-galactanase

For the galactan portion, a primary enzyme for degradation is endo-β-1,4-galactanase (EC 3.2.1.89). This enzyme specifically hydrolyzes the β-1,4 glycosidic linkage between two galactose residues in type I arabinogalactans in an endo fashion. Huisman et al. have demonstrated the utility of this enzyme in the removal of arabinogalactan sidechains from pectin polysaccharides which

were isolated by chelating agent solubilization of soybean polysaccharides (2001; 1999). In addition, Nakamura et al. have used this enzyme to release galacto-oligosaccharides from soluble soybean polysaccharides (SSPS). In particular, these results allowed them to show the presence of arabinose substituents on the galactan chains of SSPS (Nakamura et al., 2001). Ward et al. have also used this enzyme to show that exhaustive incubation of soybean arabinogalactan results in a major end product of 1,4-β-D-galactotriose (1989).

During the work for this thesis, preliminary experiments were conducted by incubating a 2.5% (w/v) soybean spent flake solution with 0.25 mg/mL endo-β-1,4-galactanase for 1 hour at 40 °C, pH 6.0. Following enzyme inactivation, analysis of the total solubilized carbohydrates in the reaction supernatant by a reducing sugar assay (briefly described in Section 6.3) showed 2.58 mM glucose equivalents released. HPAEC-PAD analysis of the monosaccharides detectable in this fraction only accounted for 42% of these carbohydrates, indicating that the majority of the carbohydrates released in this treatment were in oligomeric form. For this application, this enzyme was used to successfully solubilize a limited portion of soybean spent flakes as a proof of concept of its activity on this substrate.

exo-β-1,4-galactanase
An additional enzyme that can be used for the degradation of galactan chains is exo-β-1,4-galactanase (no EC number), which specifically hydrolyzes the β-1,4 glycosidic linkage between two galactose residues in an exo fashion. Huisman et al. have demonstrated the ability of this enzyme to remove arabinogalactan side chains from a pectin polysaccharide fraction which was isolated with chelating agent assisted solubilization of soybean polysaccharides (2001; 1999).

β-galactosidase
β-galactosidase (EC 3.2.1.23) has also shown some limited utility in the enzymatic degradation of soybean polysaccharides. This enzyme hydrolyzes terminal, non-reducing β-D-galactose residues and is notable for the fact that the glycosidic linkage of the penultimate sugar from the non-reducing end is not critical for the function of the enzyme. However, the type of linkage present can have an effect on the enzymatic rate. Nakamura et al. have shown that β-galactosidase treatment of SSPS is capable of releasing a significant amount of galactose. This result was used to conclude that the galactan chains in SSPS must be relatively absent of side chain groups, with any observed side chain moieties occurring closer to the main rhamnogalacturonan chain of the pectin backbone (Nakamura et al., 2001). These results were later validated by comparing size exclusion chromatography and light scattering measurements of SSPS and β-galactosidase treated SSPS (Wang et al., 2005).

endo-arabinanase
Endo-arabinanase (EC 3.2.1.99) catalyzes the endo hydrolysis of 1,5-α-arabinofuranosidic linkages in 1,5-linked arabinans. The linkage analysis presented in Table 5.1 shows a relatively small amount of this linkage type (2.5%) in soybean spent flakes; however, in principle this enzyme should serve to degrade these linkages. Huisman et al. demonstrated that endo-arabinanase was capable of solubilizing a relatively small quantity of soybean arabinan oligomers (1999). However, van de Vis found that this enzyme had very little activity on soybean arabinogalactan (1994). Nakamura et al. had similar results, showing that endo-arabinanase alone showed no reaction on the acidic extracted soybean polysaccharides in SSPS (2001). Overall, these results indicate limited activity of this enzyme on soybean polysaccharides.

α-L-arabinofuranosidase
α-L-arabinofuranosidase (EC 3.2.1.55) is an exo acting enzyme which catalyzes the hydrolysis of terminal non-reducing α-L-arabinofuranosidase residues. The enzyme is capable of acting on α-L-arabinans containing 1,3- and/or 1,5-linkages, arabinoxylans, and arabinogalactans. The significant quantity of observed terminal arabinose in the linkage analysis presented in Table 5.1 indicates that this enzyme should have significant activity on soybean polysaccharides. Nakamura et al. demonstrated excellent enzyme activity on the soybean polysaccharides in SSPS, obtaining a 94% release of arabinose residues from this substrate (2001). These results, in combination with their endo-arabinanase results described above, indicated that the α-1,5-linked arabinose residues in SSPS are primarily in a terminal position. This hypothesis is corroborated by the linkage analysis results from this thesis. Interestingly, Huisman et al. did not show much influence on the molecular weight distribution from α-L-arabinofuranosidase treatment of a chelating agent-soluble pectin fraction (1999). However, they did find that the enzyme released significant quantities of arabinose, results which align well with those presented in this thesis and by Nakamura et al.

6.1.2 Cellulose and xyloglucan degradation
From the linkage analysis results presented in Table 5.2, it is apparent that cellulose is found in significant quantities (23.5%) in soybean spent flakes. In addition, 2.7% xyloglucan is also observed in this material. By far, the most significant glycosidic bond that must be cleaved for the degradation of these materials is the β-1,4-glucose linkage. For this purpose, there are several key enzymes to consider. They are discussed below. Note that while the types of enzymes involved in both cellulose and xyloglucan degradation are similar, the side chain substitutions of xyloglucan often require unique enzyme specificities.

As a significant majority of soybean polysaccharide enzymatic studies have focused heavily on hemicellulosic and pectic polysaccharides, analysis of the

cellulose degradation of this material has been underrepresented in the literature. Previous research by Schols et al. demonstrated the recalcitrance of the intact soybean polysaccharides to degradation by purified enzymes and commercial enzyme mixtures (1993). For this reason, much of the enzyme research on this material has been performed on solubilized and extracted carbohydrate fractions, obtained through the methods discussed in Chapter 4. As the cellulosic polysaccharides are often left behind during this solubilization, their enzymatic degradation has not been a primary research focus. In this thesis, investigations into enzymes capable of oxidative degradation of the cellulosic soybean polysaccharides, so called lytic polysaccharide monooxygenases (LPMOs), have been a primary focus. This work is presented in detail in Chapter 7, Paper 2, and Paper 3 and has therefore been omitted from this section to avoid redundancies.

endo-β-1,4-glucanase

Endo-β-1,4-glucanase (EC 3.2.1.4), often simply referred to as cellulase, is one of the most significant enzymes in the degradation of cellulosic polysaccharides. It performs the endo hydrolysis of β-1,4-glucosidic linkages, and it is also capable of hydrolyzing the β-1,4 linkages in β-D-glucan which also contains 1,3-linkages. However, cellulose is resistant to enzymatic hydrolysis due to its crystalline and recalcitrant nature (described in Subsection 2.1.1). For this reason, endo-β-1,4-glucanase is typically utilized in enzyme blends containing cellobiohydrolase and β-glucosidase (described below). During the work for this thesis, preliminary experiments were conducted by incubating a 2.5% (w/v) soybean spent flake solution with 0.25 mg/mL endo-β-1,4-glucanase for 1 hour at 40 °C, pH 6.0. Following enzyme inactivation, analysis of the total solubilized carbohydrates in the reaction supernatant by a reducing sugar assay (briefly described in Section 6.3) showed 0.9 mM glucose equivalents released. HPAEC-PAD analysis of the monosaccharides detectable in this fraction only accounted for 40% of these carbohydrates, indicating that the majority of the carbohydrates released in this treatment were in oligomeric form. However, the amount of material solubilized with this treatment was quite low compared to the expected amount from the glucose levels in the substrate. Ouhida et al. also investigated endo-β-1,4-glucanase treatment of the unextracted solids following pectin and hemicellulose extraction, and observed very limited (10%) cellulose degradation (2002). Based on these results, they concluded that the xylans and cellulose of soybean polysaccharides are highly inaccessible to enzymatic degradation. Kasai et al. explored endo-β-1,4-glucanase digestion of soybean cellulose and were able to achieve an increase in glucose release by autoclaving the substrate to disrupt the cellulose network (2004).

glucan 1,4-β-glucosidase

Glucan 1,4-β-glucosidase (EC 3.2.1.74), also known as exo-β-1,4-glucanase, catalyzes the hydrolysis of β-1,4 linkages in glucans and gluco-oligosaccharides in an exo fashion. There have been no reported studies of its activity on soybean polysaccharides.

cellobiohydrolase I and II
Cellobiohydrolase (CBH) catalyzes the hydrolysis of β-1,4-glucosidic linkages from cellulose chains, releasing cellobiose. When the hydrolysis occurs from the reducing end of the glycan chain, the enzyme is characterized as a cellobiohydrolase I (CBH1) (EC 3.2.1.176). When the hydrolysis occurs from the non-reducing end of the glycan chain, the enzyme is characterized as a cellobiohydrolase II (CBH2) (EC 3.2.1.91). Endo-β-1,4-glucanase is thought to act on amorphous cellulose regions to open up areas for CBH attack. For this reason, these two enzymes are often used in combination.

β-glucosidase
β-glucosidase (EC 3.2.1.21) catalyzes the hydrolysis of terminal, non-reducing β-D-glucosyl residues, resulting in the release of glucose monosaccharides. The primary role of this enzyme in cellulose enzymatic degradation is thought to be its ability to relieve product inhibition of endo-β-1,4-glucanase and CBH through degradation of cellobiose (Sørensen, Lübeck, Lübeck, & Ahring, 2013). The use of this enzyme for the degradation of soybean polysaccharides is discussed in Papers 2 and 3 of this thesis (Pierce, Agger, Wichmann, & Meyer, 2017; Pierce, Agger, Zhang, Wichmann, & Meyer, 2017).

Xyloglucan specific enzymes
While endo-β-1,4-glucanase does show activity on the glucose backbone of xyloglucan, there are several specific enzymes that are directly suited for this task (Baumann, 2007). Xyloglucan specific endo-β-1,4-glucanase (EC 3.2.1.151) hydrolyzes 1,4-glucosidic linkages in xyloglucans. In addition, xyloglucan specific exo-β-1,4-glucanase (EC 3.2.1.155) hydrolyzes 1,4-glucosidic linkages in xyloglucans to remove oligosaccharides (DP 7-9) from the chain end. This hydrolysis occurs at unsubstituted glucose residues in the xyloglucan backbone. In addition, two enzymes specifically active on xyloglucan oligomers can be used in concert with these enzymes for xyloglucan degradation. Oligoxyloglucan reducing-end specific cellobiohydrolase (EC 3.2.1.150) generates substituted cellobiose from the reducing end of xyloglucans which are xylose substituted. For the enzyme to be active, the reducing end residue must be an unsubstituted glucose and the second residue must be a xylose substituted glucose (which can be further glycosylated). Oligoxyloglucan β-glycosidase (EC 3.2.1.120) catalyzes the hydrolysis of β-1,4-glucosidic linkages from the non-reducing end of xyloglucan oligosaccharides to release xylose substituted glucose residues. Lastly, α-L-fucosidase (EC 3.2.1.51) can be utilized to release terminal fructose residues from xyloglucan branching.

6.1.3 Xylan degradation
The linkage analysis presented in Table 5.2 identified that soybean spent flakes contain approximately 14.4% (glucurono)arabinoxylan. The most prom-

inent linkage associated with this polysaccharide class is the β-1,4-xylose linkage of the xylan backbone. However, due to the presence of arabinose and glucuronic acid substitutions, complete enzymatic degradation of this material often requires the use of several different enzyme activities (Sørensen, Pedersen, Jørgensen, & Meyer, 2007). The relevant enzymes for the enzymatic degradation of this polysaccharide class are listed below. Note that the tentative assignment of the xylan in soybean as (glucurono)arabinoxylan in Paper 1 is a reflection of the low abundance of glucuronic acid residues in the substrate. For this reason, it is not expected that soybean polysaccharides contain a significant amount of glucuronic acid branching on the xylan backbone.

endo-1,4-β-xylanase
Endo-1,4-β-xylanase (EC 3.2.1.8) catalyzes the endo hydrolysis of β-1,4-xylosidic linkages in xylans. This enzyme is capable of acting on xylans, arabinoxylans, and glucuronoarabinoxylans (Ward et al., 1989). Ouhida et al. have previously demonstrated limited endo-1,4-β-xylanase activity on the soybean polysaccharide residue remaining after pectin and hemicellulose extraction (2002). More recently, Ratnadewi et al. have also demonstrated some limited activity by endo-1,4-β-xylanase on a xylan fraction isolated from soybean polysaccharides (2016). In addition to this enzyme, another more specific enzyme that can be utilized for xylans containing glucuronic acid residues is glucuronoarabinoxylan endo-1,4-β-xylanase (EC 3.2.1.136), which shows a high activity towards feruloylated arabinoxylans.

1,4-β-D-xylosidase
1,4-β-D-xylosidase (EC 3.2.1.37) is an exo acting enzyme which hydrolyzes the degradation of β-1,4-linked xylose residues from the non-reducing end of the polysaccharide. It is often used in conjunction with endo-1,4-β-xylanase to aid in the degradation of arabinoxylans. Nakamura et al. have demonstrated the activity of this enzyme on xylogalacturonan isolated from SSPS and subsequently used these results to elucidate the fine structure of this extract (2002a).

α-L-arabinofuranosidase
As described in Subsection 6.1.1, α-L-arabinofuranosidase (EC 3.2.1.55) is an exo acting enzyme which catalyzes the hydrolysis of terminal non-reducing α-L-arabinofuranosidase residues. The enzyme acts on arabinoxylans, and is capable of removing 1,2- and 1,3-linked arabinose residues from the xylan backbone. The presence of these arabinose sidechains is known to slow the action of the xylan-depolymerizing enzymes described above. For this reason, it is often necessary to include α-L-arabinofuranosidase with other xylanases for the complete enzymatic degradation of arabinoxylan (Sørensen et al., 2007).

xylan α-1,2-glucuronosidase
Xylan α-1,2-glucuronosidase (EC 3.2.1.131) catalyzes the hydrolysis of α-1,2-(4-*O*-methyl)glucuronosyl residues attached to the xylan backbone. As discussed above, there is expected to be low levels of these linkages in soybean polysaccharides due to the low levels of glucuronic acid detected.

6.1.4 Rhamnogalacturonan and homogalacturonan degradation

Rhamnogalacturonan and homogalacturonan are polysaccharides found in pectin. As described in Chapter 2, homoglacturonan and rhamnogalacturonan II consist of an α-1,4-linked backbone of galacturonic acid residues. In contrast, rhamnogalacturonan I contains a backbone of the repeating disaccharide [→4)-α-D-GalA-(1,2)-α-L-Rha-(1→]. Enzymes which act on these polysaccharide classes can be roughly divided into two groups: de-esterifying enzymes and depolymerizing enzymes. De-esterifying enzymes remove the methoxyl groups in pectin to form acidic residues but do not result in material degradation. The depolymerizing enzymes consist of both hydrolases, enzymes cleaving the glycosidic bond through the addition of water, and lyases, enzymes which use β-elimination to break to glycosidic bond.

pectinesterase
Pectinesterase (EC 3.1.1.11) catalyzes the removal of the methyl ester groups on polygalacturonan chains to form galacturonic acid residues in the pectin backbone. While this does not result in the degradation of the pectin polysaccharides, it is important for the functional properties of the resulting polysaccharide. For example, SSPS that has been extracted from soybean polysaccharides can be demethoxylated through enzymatic or chemical treatment to affect the functional performance of the resulting product (Maeda et al., 1992). Huisman et al. utilized this enzyme to limited effect in the enzymatic degradation of extracted soybean pectin, with the goal of improving the activity of endo-polygalacturonase (1999).

pectin lyase
Pectin lyase (EC 4.2.2.2), also known as endo-polygalacturonate lyase, catalyzes the β-elimination cleavage of α-1,4-linked galacturonic acids in pectin. This enzyme favors the degradation of demethylesterified galacturonic acid chains (pectate) over the forms with the methyl esters still present (pectin). For polysaccharides in the methyesterified form, an alternate pectin lyase (EC 4.2.2.10) can be utilized instead (Linhardt, Galliher, & Cooney, 1987). During the work for this thesis, preliminary experiments were conducted by incubating a 2.5% (w/v) soybean spent flake solution with a 50:50 mixture of pectin lyase and endo-polygalacturonanse (described below). The enzyme mixture was dosed at 0.25 mg/mL for 1 hour at 40 °C, pH 6.0. Following enzyme inactivation, analysis of the total solubilized carbohydrates in the reaction supernatant by a reducing sugar assay (briefly described in Section 6.3) showed 1.8 mM glucose equivalents released. HPAEC-PAD analysis of the monosaccharides

detectable in this fraction only accounted for 37% of these carbohydrates, indicating that the majority of the carbohydrates released in this treatment were in oligomeric form. This is the expected result for treatment with endo acting enzymes. However, it is also interesting to note that subsequent treatment with endo-β-1,4-galactanase in combination with these enzymes increased the overall galacturonic acid released by over 20%. This suggests that removal of galactan sidechains from the rhamnogalacturonan backbone helps to boost the enzymatic degradation of the pectin. This aligns with the hypothesis presented by Nakamura et al., that neutral sidechains in soybean polysaccharides may affect the activity of pectinase enzymes in the degradation of this substrate (2000). Huisman et al. were unable to observe any significant degradation of chemically extracted soybean pectin with pectin lyase in their study (2001).

Pectate disaccharide-lyase

Pectate disaccharide-lyase (EC 4.2.2.9), also known as exo-polygalacturonate lyase, catalyzes the β-elimination cleavage of α-1,4-linked galacturonic acids from the reducing end of the polysaccharide chain. This enzyme favors the degradation of demethylesterified galacturonic acid chains (pectate) over the forms with the methyl esters still present (pectin). Yamaguchi et al. demonstrated the activity of this enzyme on hexametaphosphate extracted soybean pectin, observing release of 58% of the galacturonic acid residues in this extract (Yamaguchi, Ota, et al., 1996). Nakamura et al. have also demonstrated the utility of this enzyme in the structural elucidation of the soybean pectin polysaccharides in SSPS (2000).

endo-polygalacturonase

Endo-polygalacturonase (EC 3.2.1.15) performs random hydrolysis of 1,4-linked galacturonic acid residues. The rate and extent of hydrolysis decreases with increased degree of esterification in the galacturonan chains (Whitaker, 1990). Yamaguchi et al. investigated the activity of this enzyme on hexametaphosphate extracted soybean pectin and observed that it can be used in conjunction with exo-poly-α-galacturonosidase (discussed below) to boost pectin degradation from 26% (exo alone) to 53% (exo and endo) (Yamaguchi, Ota, et al., 1996). Nakamura et al. have also utilized this enzyme in the enzymatic degradation of SSPS for structural elucidation. They observed that treatment with endo-polygalacturonase had a significant effect on the observed molecular weight of soybean pectin polysaccharides, indicating the presence of galacturonan chains linking rhamnogalacturonan sections together (2000, 2001; 2002a, 2002b). Huisman et al. obtained contrasting results and were unable to detect significant activity of this enzyme in combination with pectin lyase, concluding that the extracted pectin fractions of their studies did not contain homogalacturonan (2001; 1999). Ouhida et al. also utilized this enzyme in the degradation of extracted pectin fractions from soybean, showing similar results to those of Nakamura et al., with significant increases in enzyme activity following removal of neutral sidechains from the pectin (2002). As described above, the results of Ouhida et al. and Nakamura et al. were confirmed

in this thesis, where it was demonstrated that the activity of a pectin lyase/endo-polygalacturonanse mixture could be further boosted through the addition of endo-β-1,4-galactanase.

exo-poly-α-galacturonosidase
There are two exo acting enzymes that can hydrolyze the α-1,4-linked galacturonic acid backbone of homogalacturonan and rhamnogalacturonan II. Exo-poly-α-galacturonosidase (3.2.1.82) does this from the non-reducing end of the polysaccharide, releasing a disaccharide of galacturonic acid. Galacturan 1,4-α-galacturonidase (EC 3.2.1.67) catalyzes the hydrolysis of the same linkage, but results in the galacturonic acid monomer. As described for endo-polygalacturonase, Yamaguchi et al. investigated the activity of this enzyme on hexametaphosphate extracted soybean pectin and observed that, when used in combination with endo-polygalacturonase (discussed above), pectin degradation is boosted from 26% (exo alone) to 53% (exo and endo) (Yamaguchi, Ota, et al., 1996). In addition, Nakamura et al. have utilized this enzyme in their structural elucidation of SSPS (2000).

Rhamnogalacturonan I specific hydrolases
For degradation of the rhamnogalacturonan I (RGI) backbone, cleavage of the [→4)-α-D-GalA-(1,2)-α-L-Rha-(1→] backbone is required. For this purpose, there are several specific enzymes that can catalyze this cleavage. Rhamnogalacturonan hydrolase (EC 3.2.1.171), also known as rhamnogalacturonase, performs endo type hydrolysis of the RGI backbone. Huisman et al. demonstrated the ability of this enzyme to degrade rhamnogalacturonan in soybean pectin extracts following saponification treatment (2001; 1999). Nakamura et al. also utilized this enzyme in the structural characterization of SSPS. They found fractions of the polysaccharides resistant to rhamnogalacturonan hydrolase treatment, indicating RGI sections that were heavily branched with neutral sidechains (Nakamura et al., 2002b). Another enzyme capable of backbone degradation of RGI is rhamnogalacturonan galacturonohydrolase (EC 3.2.1.173), which performs the exo hydrolysis of the backbone in RGI oligosaccharides to release free galacturonic acid. In addition, rhamnogalacturonan rhamnohydrolase (EC 3.2.1.174) performs exo hydrolysis of the backbone in RGI oligosaccharides to release free rhamnose from the non-reducing end of the polysaccharide. These two enzymes act on oligosaccharides, which can be generated through the use of rhamnogalacturonan hydrolase. Finally, α-L-rhamnosidase (EC 3.2.1.40) can be utilized to release L-rhamnose from the non-reducing end of partially degraded rhamnogalacturonans, as has been shown for SSPS (Nakamura et al., 2002b).

6.1.5 Commercial enzyme blends
In addition to the specific mono-component enzymes described in Subsections 6.1.1-6.1.4, a variety of commercial enzyme preparations have also been utilized in the degradation of soybean polysaccharides. In some cases, specific enzyme components from these blends were separated and purified

through chromatography, and in other cases the blends were used directly. This section will briefly highlight the commercial enzymes that have been applied to soybean polysaccharides and describe the observed activities.

Driselase® is a general cell wall degrading enzyme blend from *Basidiomycetes* sp. which contains cellulase, hemicellulase, and pectinase activities. Yamaguchi et al. demonstrated the ability of this enzyme blend to completely degrade soybean pectin (Yamaguchi, Ota, et al., 1996), and Nakamura et al. also showed its capability for extensive degradation of the pectin in SSPS (2000, 2001; 2002b). Interestingly, Huisman et al. were unable to observe any degradation from this enzyme blend when treating extracted pectin with the neutral side chains removed (2001). However, Marsman et al. observed that this enzyme blend was capable of releasing 52% of the neutral sugars and 22% of the acidic sugars from untoasted soybean meal, with similar results obtained for toasted soybean meal (1997).

Pectinex® Ultra SPL is a pectinase blend from *Aspergillus aculeatus* which contains mainly pectintranseliminase, polygalacturonase, and pectinesterase activities, with some hemicellulase and cellulase activities as well. From this enzyme blend, Huisman et al. purified an endo-β-1,4-galactanase (1999) and Nakamura et al. purified a rhamnogalacturonan hydrolase and a α-L-rhamnosidase (Nakamura et al., 2002b), all of which were used in the degradation of soybean polysaccharides as described previously. Huisman et al. were unable to detect any degradation of soybean pectin with the neutral sidechains removed (2001); however, Kasai et al. did observe effective degradation by this enzyme blend in their extensive digestion (83-85%) of autoclaved okara (2004).

Viscozyme® L is a cellulolytic enzyme blend derived from *Aspergillus* sp. which contains a variety of enzyme activities such as arabanase, cellulase, β-glucanase, hemicellulase, and xylanase. Huisman et al. tested this blend on a soybean pectin extract with the neutral side chains removed but did not observe any significant degradation (2001). However, Pérez-López et al. have used this enzyme for its cellulase, xylanase, and hemicellulase activity in order to generate low molecular weight oligosaccharides from okara during high-pressure treatment (Pérez-López, Mateos-Aparicio, & Rupérez, 2016).

Cellulase A Amano 3® is a cellulolytic blend from *Aspergillus niger*. Yamaguchi et al. fractionated this mixture to obtain an exo acting galactanase and arabinase for soybean polysaccharide degradation (Yamaguchi, Ota, et al., 1996). In addition, Nakamura et al. used this blend for the degradation of SSPS (2000).

Hemicellulase M® from *Aspergillus niger* was utilized by Nakamura et al. to isolate and purify α-L-arabinofuranosidase, endo-arabinanase, α-D-galactanase, and endo-β-1,4-galactanase for SSPS degradation (2001; 2002b).

Nakamura et al. also used this blend directly to degrade soybean pectin sidechains (2000).

Ultraflo® L is an enzyme blend from *Humicola insolens* containing primarily β-glucanase and xylanase activities. Pérez-López et al. used this enzyme for its cellulase and xylanase activities in order to generate low molecular weight oligosaccharides from okara during high-pressure treatment (Pérez-López, Mateos-Aparicio, et al., 2016).

Rapidase® Liq+ is a pectinase and hemicellulase enzyme blend which was tested by Huisman et al. on extracted pectin with the neutral side chains removed; however no activity was observed in this work (2001).

Bio-Feed Plus® is an enzyme blend containing cellulase and hemicellulase activities. Marsman et al. observed that this enzyme was capable of releasing 16% of the neutral sugars and 2% of the uronic acid sugars from untoasted soybean meal. However, for toasted soybean meal these values were significantly higher, 44% and 7% respectively (Marsman et al., 1997).

SP-249® is a multicomponent carbohydrase blend capable of degrading non-starch polysaccharides. Marsman et al. observed that this enzyme was capable of releasing 50% of the neutral sugars and 30% of the uronic acid sugars from untoasted soybean meal, with similar results observed for toasted soybean meal (1997).

Energex® is a carbohydrase blend which Marsman et al. have shown is capable of releasing 55% of neutral sugars and 22% of uronic acid sugars from untoasted soybean meal. For toasted soybean meal, the results obtained were a bit different with observed levels of 47% and 34% respectively (Marsman et al., 1997).

Biocellulase® A concentrate is a cellulase blend derived from *Aspergillus niger* and Biopectinase NKP 120P® is a commercial pectinase blend. Ouhida et al. used these blends to degrade soybean polysaccharides following sequential fractionation (2002).

Esperase®, Neutrase®, and Bio-Feed Pro® are proteolytic enzyme blends which have been tested for their ability to release the protein from soybean meal. Marsman et al. evaluated these blends and observed that Esperase®, Neutrase®, and Bio-Feed Pro® solubilized 59%, 32%, and 30% of the protein from untoasted soybean meal respectively. For toasted soybean meal, these values were 60%, 31%, and 32% respectively (Marsman et al., 1997).

6.2 Summary of previous enzymatic approaches

As discussed throughout Section 6.1, there have been a variety of enzymatic degradation experiments performed on soybean polysaccharides. While the

details and results from these experiments are described in the previous section, Table 6.1 provides a summary of this work in an effort to condense the presented information into a format which can be easily referenced.

Table 6.1 – Summary of enzymatic degradation approaches on soybean polysaccharides. Studies are identified with their reference ID, with a key provided in chronological order at the bottom of the table.

Enzyme	Reference ID(s)	Function
endo-β-1,4-galactanase	A, G, I, J, M	Degrade galactan in soybean pectin
exo-β-1,4-galactanase	G, I	
β-galactosidase	J, M, O	
endo-arabinanase	C, G, I, J, M	Degrade soybean arabinan and other arabinose-containing polysaccharides
α-L-arabinofuranosidase	G, I, J, M	
endo-β-1,4-glucanase	K, N	Degrade soybean cellulose
β-glucosidase	S, T	
endo-1,4-β-xylanase	K, P	Degrade soybean xylan
1,4-β-D-xylosidase	L	
pectinesterase	B, G	Degrade soybean pectin (homogalacturonans and rhamnogalacturonans)
pectin lyase	I	
pectate disaccharide-lyase	D, H	
endo-polygalacturonase	D, G, H, I, J, K, L, M	
exo-poly-α-galacturonosidase	D, H	
rhamnogalacturonan hydrolase	G, I, M	
α-L-rhamnosidase	M	
LPMO*	S, T	Improve soybean cellulose degradation
α-amylase	F, K, Q	Soybean starch degradation
Driselase®	D, E, H, I, J, M	General soybean cell wall degradation
Pectinex® Ultra SP	G, I, M, N	Degrade soybean pectin
Viscozyme® L	I, R	General soybean cell wall degradation
Cellulase A Amano 3®	D, H	Degrade cellulose

Hemicellulase M®	H, J, M	Degrade soybean hemicellulose
Ultraflo® L	R	Degrade soybean cellulose and xylan
Rapidase® Liq+	I	Degrade soybean pectin and hemicellulose
Bio-Feed Plus®	E	Degrade soybean cellulose and hemicellulose
SP-249®	E	General soybean cell wall degradation
Energex®	E	
Biocellulase® A Concentrate	K	Degrade soybean cellulose
Biopectinase NKP 120P®	K	Degrade soybean pectin
Proteases (Esperase®, Neutrase®, Bio-Feed Pro®)	E	Improve soybean protein extraction

Reference ID Key: **A**: (Ward et al., 1989); **B**: (Maeda et al., 1992); **C**: (Vis, 1994); **D**: (Yamaguchi, Ota, et al., 1996); **E**: (Marsman et al., 1997); **F**: (Huisman et al., 1998); **G**: (Huisman et al., 1999); **H**: (Nakamura et al., 2000); **I**: (Huisman et al., 2001); **J**: (Nakamura et al., 2001); **K**: (Ouhida et al., 2002); **L**: (Nakamura et al., 2002a); **M**: (Nakamura et al., 2002b); **N**: (Kasai et al., 2004); **O**: (Wang et al., 2005); **P**: (Ratnadewi et al., 2016); **Q**: (Pierce et al., 2016); **R**: (Pérez-López, Mateos-Aparicio, et al., 2016); **S**: (Pierce, Agger, Zhang, et al., 2017); **T**: (Pierce, Agger, Wichmann, et al., 2017); **Further described in Chapter 7.*

As is evident from Table 6.1, the most common mono-component enzymes applied to soybean polysaccharides are endo-polygalacturonase, endo-β-1,4-galactanase, and endo-arabinanase. These enzymes are logical choices, as they catalyze the degradation of some of the most abundant linkages in the material, as detailed in Table 5.1. In addition, the commercial enzyme Driselase® has been frequently applied as a generalized cell wall degrading enzyme blend due to its cellulase, hemicellulase, and pectinase activities. Table 6.1 also demonstrates the significant focus that has been placed on the soybean polysaccharide pectin fraction in past research, much of which has been driven by investigations concerning the commercial product SSPS (further described in Subsection 4.2.1). It is worthwhile to note that although the cellulosic polysaccharides of soybean are the second most abundant polysaccharide constituent in the material, representing 23.5% of the total carbohydrate content, very little research has been conducted concerning the enzymatic degradation of this material (Pierce et al., 2016). A goal of the research in this thesis was to address this shortcoming, and approaches for the degradation of this material using traditional cellulolytic enzymes and oxidizing enzymes are discussed in Chapters 7-9 as well as in Papers 2 and 3.

6.3 Other considerations

In addition to the choice of enzymes, there are several other notable considerations for the enzymatic treatment of soybean polysaccharides. A few of the most relevant considerations are discussed below.

Bacteriostatic Agents

As described in Chapter 3, soybean polysaccharides obtained directly from the manufacturing process contain a significant level of microbial contamination. When incubated under standard enzymatic treatment conditions, bacterial growth will occur and unwanted degradation of reaction products can take place. For this reason, it is often prudent to include a bacteriostatic agent in any enzymatic reactions of soybean polysaccharides. For the work presented in this thesis, sodium azide (NaN_3) was utilized for this purpose. Even at low concentrations (e.g. the 0.02% used in this work), sodium azide is capable of inhibiting cytochrome oxidase in gram-negative bacteria (Snyder & Lichstein, 1940). There are many other bacteriostatic agents that could be utilized for this purpose. In early experiments for this thesis, tetracycline was used to inhibit protein synthesis in bacteria during enzymatic reactions; however, this was replaced by sodium azide due to chemical availability. For further information, a full review of bacteriostatic compounds, their modes of action, and the advantages and disadvantages of their use has been compiled by Rezaei et al. (2012).

Use of Extractions and Sequential Treatments

As described in Section 6.1, previous research by Schols et al. has demonstrated the resistance of the intact soybean polysaccharides to degradation by purified enzymes (1993). As such, the vast majority of experiments involving the enzymatic treatment of soybean polysaccharides include a solubilization or extraction step prior to enzyme addition (Chapter 4). For example, many of the references provided in Section 6.1 used chemically extracted forms of soybean polysaccharides in their work. Nakamura et al. worked with material extracted under acidic conditions (SSPS) (2000); Yamaguchi et al. tested hexametaphosphate extracted soybean pectin (Yamaguchi, Kojima, et al., 1996); and Huisman et al. utilized chelating agent extracted soybean pectin (1998). In addition, the enzymatic work presented in Papers 2 and 3 of this thesis was conducted on NaOH pretreated soybean spent flakes (Subsection 7.2.1). Finally, certain enzymatic activities on soybean polysaccharides first require the use of additional enzymes prior to treatment. For example, Nakamura et al. describe a lack of observed activity on SSPS with pectinase enzymes unless the neutral sidechains attached to the rhamnogalacturonan are first removed, either enzymatically or chemically (2000).

Viscosity Limitations

One practical limitation when performing enzymatic treatment of soybean polysaccharides is the high viscosity and water-holding observed for this material,

even at relatively low substrate concentrations. A mixable and flowable solution can be obtained for concentrations up to roughly 6% (w/v). For slurries from 6-12% (w/v), the solution is extremely thick and can be difficult to mix properly. At concentrations above 12%, the material no longer flows and can be held in hand. This high viscosity means that enzymatic reactions, both on an industrial and laboratory scale, must be performed at relatively low substrate concentrations. This can add considerable costs to industrial enzymatic processing of this material, due to the added capacities and water removal steps required to accommodate the diluted substrate.

Analytical Techniques

The type of enzymatic reaction and composition of the degradation products dictate the precise analytical techniques required for analysis, but there are two primary techniques that are invaluable for characterization of enzyme treated soybean polysaccharide samples. The first is the ubiquitous reducing sugar assay to quantify the number of reducing ends in a sample. This technique utilizes the fact that the reducing end of a carbohydrate exists in equilibrium between the open-chain aldehyde and the ketone form, with the aldehyde form capable of acting as a reducing agent. These assays rely on this aldehyde reducing Cu(II) to Cu(I), with subsequent spectrophotometric detection. When a glycosidic bonds are broken in the substrate carbohydrate chains, additional reducing ends are exposed. This direct correlation between reducing end abundance and enzymatic activity provides a quick screening method for enzymatic activity on a substrate. The second technique is HPAEC-PAD which is further discussed in Section 2.2. This method can be used to quantify the resulting mono- and oligosaccharides from enzymatic reactions. While the utility can be somewhat limited for endo acting enzymes that do not produce small enough compounds for detection, coupling simple acid hydrolysis with this technique provides a wealth of information about the types and amounts of monosaccharides released in enzymatic reactions.

7. Lytic polysaccharide monooxygenases

While Chapter 6 discussed the application of traditional glycoside hydrolases and lyases for the enzymatic degradation of soybean polysaccharides, the aim of this chapter is to present a review of the application of lytic polysaccharide monooxygenases (LPMOs) for this purpose. The first evidence of LPMO activity on soybean polysaccharides was demonstrated in the work conducted for this thesis and is presented in Papers 2 and 3. The sections below present an overview of this class of enzymes, a description of their application to soybean polysaccharides, and an overview of the analytical methods essential for characterization of their products.

7.1 Background

7.1.1 Discovery and classification

LPMOs are a recently categorized class of oxidative enzymes capable of polysaccharide degradation. The first indication of their oxidative activity was reported by Eriksson, Pettersson, & Westermark (1974). In their research, they demonstrated that the fungus *Sporotrichum pulverolentum* displayed oxidative enzyme production that was linked to increased cellulose degradation by glucanases. In the years following this observation, other proteins, which were later confirmed as LPMOs, were classified as weakly acting endoglucanases in glycoside hydrolase (GH) family 61. Continued evidence of their ability to boost the activity of traditional GHs (Merino & Cherry, 2007) and a demonstration of the dependence of this activity on metal coordination (Harris et al., 2010) further increased the interest in these enigmatic enzymes. While it was generally agreed upon that the true function of these enzymes was beyond their limited hydrolytic activity, it was not until 2010 that Vaaje-Kolstad et al. published their work describing the oxidative mode of action and structure of CBP21 from *S. marcescens*, a chitin-active LPMO originally classified to family CBM33 (2010). In this work, a dependence was shown between LPMO activity and molecular oxygen, bivalent cations, the presence of reductants, and the presence of a crystalline substrate for CBP21 activity. Today, there is continued interest in this area, which has been motivated by the observed hydrolytic boosting effect of LPMOs on GH activity for biofuel applications (Hemsworth, Johnston, Davies, & Walton, 2015).

LPMOs are classified as auxiliary activity (AA) enzymes in the Carbohydrate-Active enZyme database (CAZy; http://www.cazy.org) (Levasseur, Drula, Lombard, Coutinho, & Henrissat, 2013). They have been classified into four families based on their activities: AA9 (formerly GH61), AA10 (formerly CBM33), AA11, and AA13. The AA9 family is comprised of fungal LPMOs active on lignocellulose. The AA10 family is predominantly composed of LPMOs of bacterial and viral origin which cleave cellulose and chitin. The AA11 family comprises fungal LPMOs that cleave chitin. Finally, the AA13

family consists of fungal LPMOs that cleave starch (Couturier, Bennati-Granier, Urio, Ramos, & Berrin, 2016). As of this writing, there are 345, 2624, 68, and 16 identified proteins in families AA9, AA10, AA11, and AA13 respectively. To date, LPMO activity has been observed for a variety of substrates including cellulose, xylan, xyloglucan, glucomannan, lichenin, starch, β-glucan, and soluble cello-oligosaccharides (Agger et al., 2014; Bennati-Granier et al., 2015; Forsberg et al., 2011; Frommhagen et al., 2015; Isaksen et al., 2014; Lo Leggio et al., 2015). In addition, the work presented in Papers 2 and 3 shows, for the first time, LPMO activity on soybean polysaccharides.

7.1.2 Oxidation types and requirements

In their original study, Vaaje-Kolstad et al. demonstrated the importance of a bivalent cation for LPMO activity but incorrectly assigned the metal cofactor as Na (2010). Additional studies towards the classification of these enzymes also incorrectly assigned the cation as Zn (Harris et al., 2010), Ni (Karkehabadi et al., 2008), and Na (Vaaje-Kolstad, Houston, Riemen, Eijsink, & van Aalten, 2005). This was due to the high binding affinity of LPMOs for copper and the difficulty in fully removing it from the system for metal-dependency studies (Hemsworth, Henrissat, Davies, & Walton, 2014). However, the characterization of the *T. aurantiacus* GH61 enzyme, *Ta*GH61, as an LPMO allowed for the conclusion that the LPMO active site utilizes copper as the coordinating metal (Quinlan et al., 2011). Vaaje-Kolstad et al. also demonstrated the importance of a reductant for the function of LPMOs. More specifically, it is now recognized that an electron source is required in order for the oxidation to take place. Most experiments utilize small-molecule reductants *in vitro*, such as ascorbate or cysteinate. Cellobiose dehydrogenase (CDH) can be utilized for some LPMOs in an effort to mimic *in vivo* conditions, as this enzyme is capable of catalyzing the two electron oxidation of cellobiose and providing electrons for the LPMO to function (Phillips, Beeson, Cate, & Marletta, 2011). Additionally, it has been shown that lignin present in the polysaccharide substrate can act as an electron donor to activate LPMOs (Dimarogona, Topakas, Olsson, & Christakopoulos, 2012). While it has been established that the overall LPMO reaction pathway requires two electrons per cycle, the exact mechanism is still under debate. The general understanding is that one of these electrons results from the conversion of Cu(II) to Cu(I). The second electron has been proposed to result from internal electron transportation through the substrate-bound LPMO (Walton & Davies, 2016); however recent work has proposed that this electron comes from Fenton-type H_2O_2-derived hydroxyl radicals (Bissaro et al., 2016).

There are two primary ways in which LPMOs can oxidatively cleave the 1,4-linked glycosidic bonds in their respective substrates. Figure 7.1 depicts these oxidations for a β-1,4-linked cellulose polymer. For C1 oxidation, the oxygen is added at the C1 position of the reducing end of the resulting product. This directly forms a reducing-end lactone, which readily hydrates in an aqueous environment to form a reducing-end aldonic acid. For C4 oxidation, the oxygen

is added at the C4 position of the non-reducing end of the resulting product. In this case, a non-reducing end ketoaldose is formed, which is also capable of hydrating to form a non-reducing end gemdiol. Interestingly, the end result of both types of oxidation is a mass shift of +16 Daltons over a non-oxidized oligo- or polysaccharide of the same size. This creates challenges for the analytical differentiation of these oxidation products, as further described in Section 7.3. It should be noted that LPMO oxidation at the C6 position has also been described in the literature (Bey et al., 2013; Quinlan et al., 2011). In these studies, the AA9 LPMOs from *T. aurantiacus* (*Ta*GH61A) and *P. anserine* (*Pa*GH61B) were categorized as C6 oxidizing based on mass spectrometry results. However, additional attempts to confirm this activity with NMR spectroscopy have only shown C4 oxidation (Isaksen et al., 2014). While it still remains to be seen whether C6 oxidation is possible, it has been omitted from further discussion in this work as it is a non-productive oxidation pathway which does not result in oxidative cleavage of the glycosidic backbone.

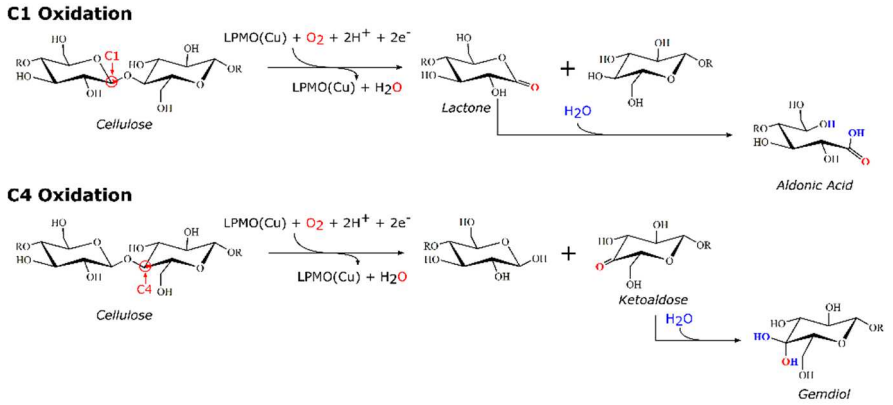

Figure 7.1 - LPMO oxidative cleavage of β-1,4-linked glucose units in cellulose. C1 oxidation results in a lactone which can be hydrated to form an aldonic acid. C4 oxidation results in a ketoaldose which can be hydrated to form a gemdiol.

7.1.3 Regioselectivity and structure

The four different families of LPMOs described above have also shown differences in oxidative regioselectivity. AA9 LPMOs have shown both C1 and C4 oxidation and have thus been classified into three types: type 1 (C1 oxidizing), type 2 (C4 oxidizing), and type 3 (both C1 and C4 oxidizing) (Vu, Beeson, Phillips, Cate, & Marletta, 2014). In contrast, the AA10 family LPMOs show primarily C1 oxidation. While evidence of double oxidation has been reported for AA10 LPMOs (Forsberg et al., 2014), there is currently no evidence of C4 oxidation alone. The AA11 and AA13 families are more recently classified but to date have only displayed C1 oxidized products.

There has been significant interest in understanding the cause of this observed regioselectivity for LPMOs and, in particular, to link this regioselectivity

with other attributes of the protein (Vaaje-Kolstad, Forsberg, Loose, Bissaro, & Eijsink, 2017). It was originally suggested that the classification of LPMOs into the three types described above could be accomplished directly with sequence-based classification (Vu et al., 2014). In particular, studies of the crystal structures of several AA9 LPMOs showed conserved structural features that could be linked to sequence data and attributed to certain oxidation types. However, recent studies have suggested that this approach may not always be accurate in its prediction of oxidative regioselectivity. For example, the recently characterized type 3 LPMO, *Pa*AA9_H, was originally predicted to be a type 2 LPMO from this sequence-based approach (Bennati-Granier et al., 2015). While there is still more work to be done in this area, these preliminary studies reveal that regioselectivity may not be fully predictable by sequence data alone. Additional studies on the nature of the copper active site utilizing AA10 LPMOs have also suggested correlations to regioselectivity. In particular, a conserved Ala residue in AA10 LPMOs, whose position is capable of affecting the ligand coordination at the copper active site, has been investigated (Hemsworth et al., 2013). These studies suggest that this amino acid affects the ability of copper to coordinate with a ligand in the axial position and thereby affects whether C1 or C4 oxidation is observed. In this respect, structural information and modeling of the ligand binding at the copper active site may allow for accurate predictions about the oxidative regioselectivity in LPMOs (Frandsen & Lo Leggio, 2016).

A structural feature of all characterized LPMOs is the presence of a histidine brace at the enzyme active site. Figure 7.2 depicts a schematic representation of this site for a typical AA9 LPMO. This brace is primarily comprised of the amino group of the N-terminal histidine, the imidazole side chain of the N-terminal histidine, and the imidazole side chain of an additional histidine. Structural characterization of LPMOs from families AA9, AA10, AA11, and AA13 have shown a strong conservation of this basic histidine brace (Frandsen & Lo Leggio, 2016). In addition, all characterized AA9, AA11, and AA13 LPMOs feature a Tyr residue that may coordinate, through its hydroxyl group, to the copper as well. In AA10 LPMOs, a Phe residue is often found in place of this Tyr (Vaaje-Kolstad et al., 2005), and an additional Ala adjacent to the non-terminal histidine in the histidine brace also coordinates to the copper (Hemsworth et al., 2013). It should be noted that overall, AA9 LPMOs display quite limited similarities in sequence identity across the family. An additional characteristic feature of LPMOs is the presence of the solvent exposed active site at a planar surface of the protein instead of its presence in a cleft or binding pocket more typical of many GHs (Morgenstern, Powlowski, & Tsang, 2014). The enzyme utilizes aromatic sidechains near the active site to coordinate with the substrate in a similar manner to how carbohydrate-binding modules (CBMs) function (Li, Beeson, Phillips, Marletta, & Cate, 2012). This flat surface active site allows for the enzyme to act on crystalline and highly coordinated substrates such as cellulose, where binding to a single cellulose chain is made extremely difficult due to the hydrogen binding amongst the multiple chains, as described in Subsection 2.1.1.

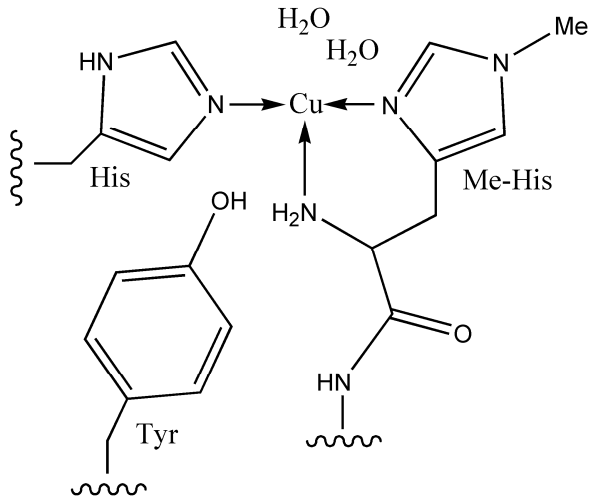

Figure 7.2 - The LPMO histidine brace for a typical AA9 LPMO expressed in filamentous fungi. Note the presence of a Tyr that may participate in the Cu coordination and the methylation of the N-terminal histidine (Frandsen & Lo Leggio, 2016).

LPMOs that have been expressed in filamentous fungi often display a characteristic Nε methylation of the N-terminal histidine imidazole nitrogen (shown in Figure 7.2). This modification is of interest because of the role of this N-terminal histidine in the copper coordination at the enzyme active site. Currently, the exact role and function of this modification remains unclear. This is due to the fact that many fungal LPMOs which have been expressed in systems incapable of performing this modification still show activity without this methylation. Computational studies have indicated that this methylation plays no significant role in LPMO activity (Kim, Ståhlberg, Sandgren, Paton, & Beckham, 2014); however it has also been suggested that the presence of this methylation could enhance electrostatic interaction between the enzyme and the substrate, subsequently affecting the activity or affinity of the enzyme (Frandsen et al., 2016).

7.1.4 Hydrolytic boosting

One of the driving forces behind the interest in LPMOs this past decade has been their demonstrated ability to boost the overall yields obtainable with traditional GHs. In 2007, Merino & Cherry described that the addition of an LPMO protein (then classified as GH61) had a significant impact on the saccharification of acid pretreated corn stover. Dosage of the LPMO at only 5% of the total enzyme load was able to maintain the same level of degradation with a twofold reduction in the cellulase enzymes (2007). In addition, Harris et al. reported a twofold increase in the saccharification of pretreated corn stover with the addition of GH61 proteins, later to be classified as LPMOs (Harris et al., 2010). These observations continued, with Langston et al. demonstrating an eightfold

increase in phosphoric acid swollen cellulose (PASC) degradation to monosaccharides with the addition of the *T. terrestris* GH61 (2011).

The prevailing hypothesis for this observed hydrolytic boosting has been that the LPMOs serve to introduce new chain breaks in crystalline or recalcitrant substrates, thereby allowing traditional GHs to act on otherwise inaccessible portions of the substrate (Horn, Vaaje-Kolstad, Westereng, & Eijsink, 2012). The inclusion of LPMOs allows for an updated view of the enzymatic degradation of polysaccharides. For cellulose, C1 and C4 oxidizing LPMOs would generate additional non-oxidized ends for cellobiohydrolase (CBH) to act upon. CBH would then generate cellobiose for subsequent conversion to glucose monosaccharides by beta-glucosidase. In addition, the oxidative cleavages resulting from LPMO activity impact the crystallinity of the substrate and expose additional surface area of the cellulose for cellulase degradation (Eibinger et al., 2014). This hydrolytic boosting effect has been demonstrated for a wide variety of substrates such as cellulose (Langston et al., 2011), lignocellulose (Merino & Cherry, 2007), spruce (Dimarogona, Topakas, & Christakopoulos, 2013), and kenaf and oak (Jung, Song, Kim, & Bae, 2015). The work presented in Papers 2 and 3 of this thesis also demonstrates the hydrolytic boosting effect for multiple LPMOs on soybean polysaccharides (Pierce, Agger, Wichmann, et al., 2017; Pierce, Agger, Zhang, et al., 2017).

7.2 Use on soybean polysaccharides

The linkage analysis results presented in Paper 1 and Tables 5.1 and 5.2 demonstrate that soybean polysaccharides are composed of a variety of polysaccharide classes. Cellulose, representing 23.5% of soybean spent flakes, is the second most abundant class. The chemical solubilization approaches presented in Chapter 4 and the enzymatic solubilization approaches presented in Chapter 6 focused primarily on the non-cellulosic soybean polysaccharides, namely the pectin and hemicellulose fractions. An early observation in this PhD project was the apparent link between the insolubility of soybean polysaccharides and the cellulose content of the polysaccharides. It was noted that all solubilization approaches serve to separate the more labile and soluble polysaccharide classes from the recalcitrant and insoluble cellulosic material. For example, the residual insoluble residue resulting from the peroxide solubilization process described in Subsection 4.2.2 is heavily enriched in 1,4-linked glucose (Pierce et al., 2016). The same holds true for the unextracted solids resulting from the SSPS process (Figure 4.1). In addition, the acid hydrolysis methods for monosaccharide determination described in Subsection 2.2.1 can be challenging to adapt to soybean polysaccharides due to the resistance of the soybean cellulose to degradation (further described in Section 3.5). These observations help to confirm Hypothesis 1 of this thesis (Section 1.2): the insolubility of soybean polysaccharides is linked to the structural make-up of the carbohydrates. As described in Subsection 6.1.2, efforts to enzymatically degrade the cellulose material in soybean spent flakes with traditional enzymes were relatively unsuccessful. For that reason, investigations

into LPMO activity on soybean polysaccharides were conducted. These results are presented in more detail in Papers 2 and 3. In this section, additional considerations of the LPMO treatment of soybean polysaccharides will be discussed.

7.2.1 Pretreatment

For the research presented in this thesis, initial attempts to investigate LPMO activity on soybean polysaccharides involved direct treatment of soybean spent flakes. However, no activity could be observed, despite investigation of twenty-four unique LPMOs at high dosage levels (Pierce, Agger, Zhang, et al., 2017). This lack of activity on the native material was first observed for the *Trichoderma reesi* LPMO, *Tr*Cel61A, described in Paper 2. This enzyme showed excellent oxidative degradation of an amorphous cellulose sample, phosphoric acid swollen cellulose (PASC), but no ability to degrade crystalline cellulose, Avicel® PH-101. It has previously been shown that the crystallinity of cellulose is one of the most important influences on its enzymatic digestibility by traditional glycoside hydrolases (Fan, Lee, & Beardmore, 1980; Hall, Bansal, Lee, Realff, & Bommarius, 2010). For this reason, it was hypothesized that the cellulose crystallinity in the native soybean spent flakes could be the property limiting LPMO activity on this substrate, and chemical treatments to reduce cellulose crystallinity were investigated.

The ability of alkaline treatment to affect the transformation of cellulose from the cellulose I allomorph to the cellulose II allomorph has previously been shown (Fengel, Jakob, & Strobel, 1995). In addition, Mittal et al. investigated the effects of this alkaline treatment on cellulose crystallinity and enzymatic digestibility (2011). They found that NaOH treatment successfully formed the cellulose II allomorph, resulted in lower cellulose crystallinity, and produced the sample most readily degraded by enzymatic digestion. Based on these successful results, this treatment was performed in this thesis on soybean spent flakes, using 0.857 mg /mL spent flakes in 16.5% (w/w) NaOH at 25 °C for 2 hrs. Figure 7.3 shows the X-ray powder diffraction spectra obtained for the native and NaOH pretreated materials, using $CuK\alpha_1$ radiation as previously described by Thygesen et al. (2005).

Figure 7.3 depicts the change from the cellulose I into the cellulose II allomorph, denoted by the appearance of a doublet at 2θ values of 20° and 22°. Unfortunately, the heterogeneity of the polysaccharide constituents of the spent flakes and the lack of analogous amorphous control samples makes it difficult to accurately measure the crystallinity through X-ray diffraction (Thygesen et al., 2005) or NMR (Park, Johnson, Ishizawa, Parilla, & Davis, 2009). Nonetheless, it appears that the alkaline treatment is acting in the expected manner as outlined by Mittal et al. (2011).

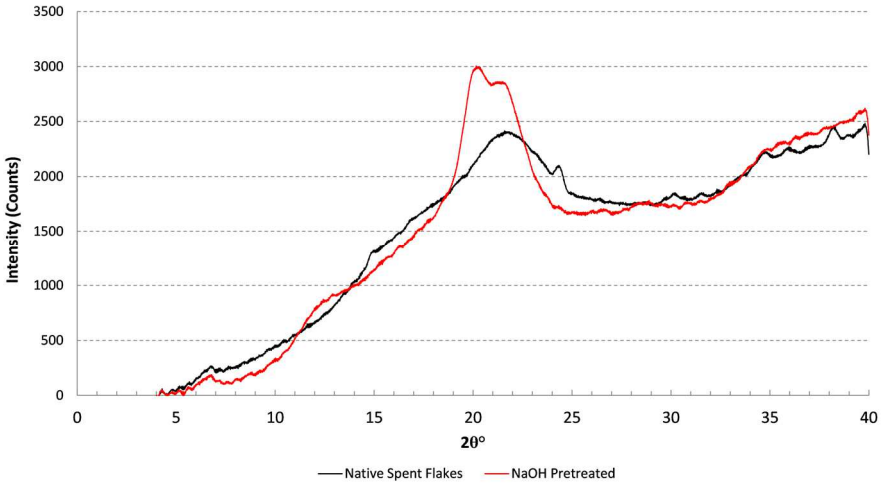

Figure 7.3 - X-ray powder diffraction spectra obtained for native soybean spent flakes (black) and NaOH pretreated soybean spent flakes (red). Pretreatment conducted as described in Pierce, Agger, Wichmann, et al. (2017) and X-ray diffraction conducted with CuKα_1 radiation as described by Thygesen et al. (2005).

As described in Paper 2, the NaOH pretreatment employed also affects the carbohydrate composition of the soybean spent flakes. To prevent redundancy, the full results will not be duplicated here; however, it is worthwhile to note the changes observed in constituent monosaccharides. The native spent flakes contain galactose (34.7%), arabinose (20.7%), glucose (19.1%), galacturonic acid (11.3%), xylose (8.4%), fucose (3.0%), and rhamnose (2.8%). The NaOH pretreated spent flakes consist of glucose (51.8%), galactose (15.3%), arabinose (12.6%), galacturonic acid (10.8%), xylose (6.1%), fucose (1.6%), and rhamnose (1.6%). From these results, it is immediately apparent that the NaOH pretreated material has significantly less galactose and arabinose and is much more enriched in glucose. As discussed in detail in Subsection 4.1.3, alkaline treatment of cell wall material is capable of solubilizing hemicellulose and pectin polysaccharides. The conditions employed in this pretreatment, 4.1 M NaOH, are analogous to the 4 M alkaline treatments utilized in these chemical extraction studies. These extractions also resulted in a significant solubilization of soybean pectin sidechains consisting of galactose and arabinose and a subsequent enrichment of the residual material in cellulosic polysaccharides. With this understanding, we see that the true effect of the NaOH pretreatment is two-fold: conversion of the cellulose to the cellulose II allomorph and removal of hemicellulose and pectin polysaccharides to enrich the spent flakes in the cellulosic components.

The results presented in Paper 3 show that seven of the LPMOs investigated display activity on NaOH pretreated spent flakes (Pierce, Agger, Zhang, et al., 2017). This activity may be the result of changes in the cellulose conformation, but it is also expected that the removal of the hemicellulose and pectin polysaccharides allows for increased substrate access by the LPMOs. In total,

these results illustrate the necessity for material pretreatment in order to observe LPMO activity on soybean polysaccharides.

7.2.2 Galactose content

Another consideration in the LPMO treatment of soybean polysaccharides is the high content of galactose present in the substrate. To date, there have been no reports of LPMO activity on the β-1,4-linked galactose residues in galactan. However, the strong dependence on mass spectrometry for analysis of LPMO reaction products (discussed in Subsection 7.3.1) means that a risk exists for misinterpretation of results due to the identical masses of galactose and glucose residues. Currently, the only example of an LPMO acting on a polysaccharide linkage other than 1,4-linked glucose residues is the xylan degrading LPMO, *Mt*LPMO9A, from *Myceliophthora thermophila* C1 (Frommhagen et al., 2015). It is worthwhile to note that, in this study, *Mt*LPMO9A only showed oxidative activity on xylan in the presence of cellulose.

For the LPMO reactions conducted in this thesis and presented in Papers 2 and 3, control samples of purified galactan were tested in tandem with soybean polysaccharides for LPMO reactivity. While no oxidation of galactan could be observed, the work of Frommhagen et al. has established that a lack of activity on the purified polysaccharide does not preclude the presence of activity on the complex native substrate. For this reason, additional investigations with high-performance anion exchange chromatography in combination with beta-glucosidase were conducted to verify that the observed oxidized reaction products were composed of glucose (further described in Subsection 7.3.2). While it is unlikely that any oxidized products observed from LPMO treatment of soybean polysaccharides are a result of oxidized galacto-oligosaccharides, it is nonetheless important to include the proper controls and experiments to confirm the experimental findings for this complex substrate.

7.2.3 Applications

Papers 2 and 3 demonstrate the activity of seven LPMOs on NaOH pretreated soybean spent flakes. As an example of the utility of these enzymes to boost the enzymatic degradation of soybean polysaccharides, these studies present hydrolytic boosting experiments testing the combination of each LPMO with endo-β-1,4-glucanase. The results show the ability of the top performing LPMOs to increase the amount of glucose released by endoglucanase by over 75% for the same reaction time (Pierce, Agger, Zhang, et al., 2017). This finding is significant for the saccharification of soybean polysaccharides to monosaccharides, which could subsequently be utilized in biofuel applications. Traditionally, the fermentation of glucose to ethanol is the primary pathway for bioethanol production from cellulosic biomass (Olofsson, Bertilsson, & Lidén, 2008). As discussed in Chapter 3, the primary monosaccharide constituents of soybean polysaccharides are galactose, arabinose, and glucose, which

represent almost 75% of the carbohydrate content of the material. Recent work to investigate organisms capable of arabinose and galactose fermentation have shown some promise for the utilization of hemicellulose-rich feedstocks for biofuel applications (Kim, Ryu, et al., 2014; Kuhad, Gupta, Khasa, Singh, & Zhang, 2011; Richard, Verho, Putkonen, Londesborough, & Penttilä, 2003). While these findings could result in improved utilization of soybean polysaccharides in biofuel applications, it is currently more likely that the saccharification of soybean polysaccharides could be used for the purification and isolation of the constituent monosaccharides for alternate applications.

The work presented in this thesis demonstrates the ability of LPMOs to boost the enzymatic degradation and accessibility of soybean polysaccharides. This activity may give a further understanding of the relationship between the cellulosic and hemicellulose polysaccharides in soybean, something that has been absent from previous structural studies with chemically extracted fractions (Section 4.1). It may also open alternate possibilities for the solubilization of this recalcitrant material, thereby increasing its utility in food applications and the ability of analytical techniques to characterize its structure.

7.3 Analytical methods for product characterization

Analytical methods for the characterization of LPMO reaction products are essential in the determination of LPMO reactivity, substrate specificity, and types of oxidation. The work presented in this thesis primarily utilized mass spectrometry and high-performance anion exchange chromatography for the characterization of LPMO reaction products (Papers 2 and 3). These techniques are discussed in greater detail below; however, additional analytical methods currently used in LPMO product characterization have also been briefly described here for completeness. The intention of this section is not to describe the fundamental aspects of these analytical techniques, but instead to address the advantages and disadvantages of each method for LPMO product characterization.

7.3.1 Mass spectrometry

The use of mass spectrometry (MS) in the characterization of LPMO reaction products has been widespread since the first identification and description of LPMO oxidative capabilities. For preliminary studies, MS is particularly useful for the fast and direct determination of whether oxidation took place following LPMO treatment.

As described in Subsection 7.1.2, LPMO oxidation of polysaccharides can occur as C1 oxidation, C4 oxidation, or a combination of the two. If we consider a non-oxidized oligomer of mass M Da, we observe that the C1 oxidized version of this oligomer could potentially result in two observable mass shifts: [M-2] Da for the reducing end lactone or [M+16] Da for the aldonic acid form. In the same manner, a C4 oxidized version of this oligomer could give two mass

shifts as well: [M-2] Da for the non-reducing end ketoaldose or [M+16] Da for the gemdiol form. These mass shifts make it readily apparent if oxidation has occurred; however, their symmetry makes determination of the types of oxidation more challenging. One indication that the [M+16] Da signal corresponds to the C1 oxidized aldonic acid over the C4 oxidized gemdiol is evidence that the aldonic acid proton has been displaced by the buffer counterion. For example, in a Na buffered system a signal at [M+38] Da can be observed, corresponding to the $[M+16+Na]^+$ ion.

MALDI-TOF

Matrix-assisted laser desorption/ionization with time of flight detection (MALDI-TOF) is an excellent tool for identification of LPMO oxidation, and in fact it was the method used by Vaaje-Kolstad et al. to first identify LPMO oxidation products (2010). It is also quite simple and straightforward to use, making it ideal for the quick screening of LPMO reaction products. MALDI is well suited for this screening work because it is generally less susceptible to interferences by buffer components or contaminants when compared to other mass spectrometry techniques, and it also allows for a wide mass detection range (Signor & Erba, 2013). However, the experiments conducted for this thesis demonstrated that sodium buffers, such as sodium acetate, were preferable when compared with phosphate buffers for MALDI analysis of LPMO oxidation products.

The sample preparation for MALDI involves combining the enzymatically treated samples with a matrix (e.g. 2,5-dihydroxybenzoic acid) and drying. The low amount of water present for this analysis results in an increased proportion of the non-hydrated forms of the oxidized products, most notably the C4 oxidized ketoaldose although this is also the case, to a lesser extent, for the C1 oxidized lactone. For this reason, it is common to observe strong [M-2] Da peaks for LPMO oxidized samples analyzed with MALDI. The disadvantage of MALDI analysis is the lower mass resolution and generally higher baseline noise when compared to other MS techniques. This lack of mass resolution means that it can be difficult to discern very similar masses, such as the K-adduct of a non-oxidized oligomer compared with the double sodiated and singly charged C1 oxidized product, $[M+16+2Na-H]^+$. Both of these compounds have a mass of [M+38] and can lead to confusion when using substrates containing potassium. The baseline noise can be lessened to some extent by utilizing only the minimal laser energy required to ionize the samples. However, the optimal energy level will vary based on sample abundance and the buffer system used, so must therefore be discerned through trial and error. Examples of the application of MALDI-TOF for LPMO product characterization are given in Paper 2.

ESI MS-MS

Electrospray ionization mass spectrometry (ESI-MS) can be utilized with direct-infusion to give similar results to those described for MALDI-TOF. A key difference between these techniques is that ESI-MS analysis takes place in

an aqueous environment, thus the oxidation products are driven to their hydrated forms, namely the C1 oxidized aldonic acid and the C4 oxidized gemdiol. Another advantage of ESI-MS is that significantly higher mass resolution can be obtained when compared with MALDI-TOF, depending on the equipment used. In addition, the baseline resolution can be greatly increased with this technique. However, the true power of this technique is attained through the use of tandem mass spectrometry (MS-MS). While it is also possible to use liquid chromatography (LC) separation prior to MS analysis, it is often not required due to the use of MS-MS analysis to identify oxidation types as described below.

Due to their identical masses, it is impossible to conclusively determine if LPMO oxidation occurs at the C1 or C4 position through the oxidized mass alone. However, MS-MS fragmentation allows for the determination of the location of the oxidation and subsequent identification of the type of oxidation. This application of MS-MS to identify C1 and C4 oxidation has been well described by Isaksen et al. (2014), and it was utilized for the work described in this thesis. Figure 7.4 depicts the sodiated masses of the expected fragmentation products from an oxidized cellobiose disaccharide. While modified versions of this figure have been included in Papers 2 and 3, it has also been reproduced here in a more detailed form to aid in the discussion of this method.

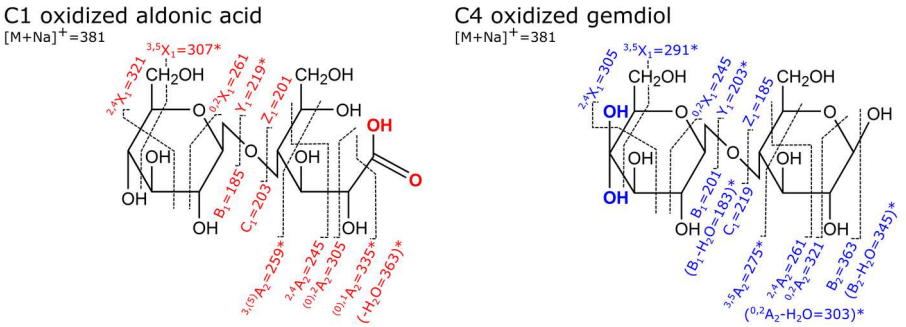

Figure 7.4 - MS-MS fragmentation products (sodium adducts) of the C1 oxidized disaccharide (aldonic acid - red) and the C4 oxidized disaccharide (gemdiol - blue). Fragments characteristic of either C1 or C4 oxidation are highlighted by an asterisk.

Examining Figure 7.4, it is very apparent that symmetry between the C1 and C4 oxidized products results in many fragments of identical mass. For example, both compounds will fragment into *m/z* 363, 321, 305, 261, 245, 219, 203, 201, and 185. Distinct MS-MS fragments for the C1 oxidized aldonic acid are m/z 335, 307, and 259, while distinct MS-MS fragments for the C4 oxidized gemdiol are *m/z* 345, 291, and 275. In addition, both compounds feature some discrete ions and signal intensities that are strong indicators of their presence. During MS-MS fragmentation, the C1 oxidized aldonic acid will show a strong *m/z* 335 signal, a relatively high *m/z* 219 signal, and a *m/z* 173 signal. The C4 oxidized gemdiol will show evidence of two water losses at *m/z* 363 and 345,

a relatively high *m/z* 303 signal, and a *m/z* 183 signal resulting from water loss of the B_1 species. These indicators can be used, in combination with the techniques described below, to elucidate the types of oxidation resulting from LPMO treatment. Examples of the application of ESI MS-MS for LPMO product characterization are given in Papers 2 and 3.

7.3.2 High-performance anion exchange chromatography

High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is a common HPLC method for the separation and characterization of carbohydrates (Rocklin & Pohl, 1983). Applications of this technique were developed by Westereng et al. for the characterization of LPMO oxidation products (2013). HPAEC is well suited for this task due to the strong retention of compounds containing an acidic group, such as the C1 oxidized aldonic acid. In addition, the PAD detector provides high sensitivity capable of measuring the often-low levels of oxidized products produced during LPMO treatment. Most importantly, HPAEC-PAD has an advantage over the MS analyses described above as it allows for the potential quantification of LPMO oxidation products. In order to utilize HPAEC-PAD as a quantitative technique, oxidized cello-oligosaccharide standards are required. These are not readily available commercially; however, they can be produced and purified using various techniques. For example, C1 oxidized oligomers can be produced from non-oxidized oligomers through selective C1 oxidation by iodine (Forsberg et al., 2011) and subsequent fractionation using porous graphitic carbon chromatography (PGC). C4 oxidized oligomers can be directly generated through LPMO treatment. Beta-glucosidase (BG) treatment can then be used to selectively degrade the non-oxidized and C1 oxidized oligomers. This leaves only the C4 oxidized products in oligomeric form, which can then be fractionated using PGC (Westereng et al., 2016). This is possible due to the fact that BG acts on the non-reducing end of the oligosaccharides and is inhibited by the presence of the gemdiol group in the C4 oxidized products.

While the analysis and quantification of C1 oxidized products is straight-forward with this technique, the analysis of C4 oxidized products is more challenging. Under the alkaline conditions employed using HPAEC, keto-saccharides may undergo tautomerization and chemical modification (Isaksen et al., 2014). This potential for on-column decomposition of C4 oxidized products highlights the limitations of this technique for the characterization and, more notably, the quantification of these compounds. Another disadvantage of this technique is the difficulty in utilizing MS detection due to the high salt and high pH conditions. While it is possible to use post-column ion removal and neutralization prior to MS detection, this can be challenging experimentally and often results in poor sensitivity. In addition, very late eluting compounds corresponding to products with both C1 and C4 oxidation require high-salt elution conditions and cannot be identified with MS due to desalting limitations.

Standard chromatography conditions for HPAEC-PAD analysis of LPMO oxidized products results in four distinct signal groupings. Non-oxidized native oligomers elute first, C1 oxidized compounds elute second, C4 oxidized compounds elute third, and C1/C4 doubly oxidized compounds elute last. While it would typically be expected that the C4 oxidized compounds would elute prior to the C1 oxidized compounds, the on-column tautomerization and modification of the C4 compounds described above results in later elution times. The four signal groups described above often show some degree of overlap in the chromatography. In order to confirm the presence of C4 oxidized compounds in this thesis, a combination of enzymatic treatment and HPAEC-PAD was employed. Standard LPMO reactions were conducted on PASC, and signals were observed in the native, C1, C4, and C1/C4 regions of the HPAEC-PAD chromatogram. The samples were then incubated with BG in order to hydrolyze all non-C4 oxidized compounds as described above. Following this treatment, signals corresponding to native and C1 oxidized products were removed from the chromatogram and the presence of C4 oxidation could be confirmed. Applications of HPAEC-PAD for LPMO product characterization are further described in Papers 2 and 3.

7.3.3 Additional techniques

The following methods were not utilized for the work presented in this thesis, but they have advantages and disadvantages when compared with the MS and HPAEC methods described above.

PGC

Porous graphitic carbon chromatography (PGC) is a useful chromatographic tool for LPMO product characterization (Westereng et al., 2016). In principle, the PGC column separates compounds based on polarity and molecular planarity (Bassler & Hartwick, 1989). In practice, this means that the C1 oxidized compounds, containing an acidic functional group, elute first followed by the non-C1 oxidized species. In addition, the strong affinity of the stationary phase for longer chain oligomers (> DP5) means that only DP2-5 oxidized species will elute and be evaluated with this technique. However, a limitation of this technique is the limited separation obtained between C1 oxidized and C1/C4 oxidized species, as well as between non-oxidized and C4 oxidized species. While this resolution does improve with increased DP, MS detection or enzymatic removal of certain oxidation products (e.g. the BG treatment described above) is typically required to identify these co-eluting components. However, the slightly alkaline conditions (pH 8.0) and lower salt concentration make this technique well suited for MS analysis, providing an advantage over HPAEC-PAD analysis in this respect.

HILIC

Hydrophilic interaction liquid chromatography (HILIC) utilizes polar stationary phases, such as those used in normal phase liquid chromatography, in combination with mobile phases similar to those used in reversed-phase liquid

chromatography. This technique allows for the efficient chromatographic separation of small polar compounds (Buszewski & Noga, 2012). Applications of this technique for LPMO product characterization have demonstrated its utility in the analysis of C1 oxidized aldonic acids (Westereng et al., 2013). However, the elution conditions required in this analysis are not ideally suited for MS analysis. Recently, alternative HILIC stationary phases employing weak anion exchange properties were investigated for separation of LPMO oxidation products (Westereng et al., 2016). These results suggested that C1 oxidized components could be separated from non-C1 oxidized compounds; however, no separation was observed between C4 oxidized and native non-oxidized oligomers. In addition, C4 oxidation peaks displayed broad bands that may be the result of an equilibrium between the keto- and gemdiol forms of the product under the chromatographic conditions employed. In total, HILIC is useful for characterization of C1 oxidized fragments but does not currently show utility for C4 oxidized compounds or for samples with mixtures of oxidation types.

Dynamic Viscosity

A method for monitoring LPMO activity through measurement of viscosity reduction has recently been reported by Kojima et al. (2016). While this technique does not allow for identification of the oxidation types or investigations of the regioselectivity of LPMOs, it does present a technique for monitoring LPMO reactivity in complex systems. Viscosity can be calculated from measurements of the rolling time of a ball through a liquid capillary. This data can then be used to monitor the depolymerization rates in the reaction system through indirect measurements of the numbers of cuts introduced into the polysaccharide chain. This technique is particularly useful for rapid screening of LPMO activity on a variety of substrates and, in some cases, it is able to detect activities that the HPLC and MS methods discussed above cannot. An additional advantage of this method is the immediate applicability of the viscosity results for industrial biomass processing.

NMR

NMR structural characterization of carbohydrates was briefly discussed in Section 2.2. Applications of these techniques for characterization of LPMO oxidation products, including determination of C1 and/or C4 oxidation, have been described in the literature (Isaksen et al., 2014). With regards to LPMO oxidized product characterization, two NMR techniques are particularly useful. HSQC is able to provide a quick visualization of the occurrence of oxidations following LPMO treatment. This is the result of the changes to carbon chemical shifts as a result of oxidation. In addition, the HMBC spectra can also readily identify the presence of both the C4 oxidized ketone and gemdiol forms. Using the signal intensities for these two compounds, it is possible to evaluate the relative ratio of these oxidized forms in a given sample. Due to the similarities in masses and MS-MS fragmentation profiles for LPMO oxidized compounds, NMR is a particularly powerful tool for definitive assignment of the structure of these products.

8. Paper 2: Oxidative cleavage and hydrolytic boosting of cellulose in soybean spent flakes by *Trichoderma reesei* Cel61A lytic polysaccharide monooxygenase

Pierce, B. C.; Agger, J. W.; Wichmann, J.; & Meyer, A. S. 2017. Enzyme and Microbial Technology, 98, 58-66.

This chapter contains a discussion of Paper 2 and its role in the work presented in this thesis. The full text of this paper can be found in the Publications section at the end of this thesis.

Brief summary
Paper 2 presents an investigation of the activity of *Tr*Cel61A, a lytic polysaccharide monooxygenase (LPMO) from *Trichoderma reesei*, on soybean spent flakes. An examination of the substrate specificity of this enzyme is conducted utilizing cello-oligomers, galactan, amorphous cellulose, crystalline cellulose, and soybean polysaccharides. In addition, NaOH pretreatment is investigated for its ability to improve the enzymatic degradation of soybean polysaccharides. With this pretreatment, the first demonstrated evidence of LPMO activity on soybean polysaccharides is described, and the resulting oxidation products are characterized with HPAEC-PAD and MS. Lastly, the ability of LPMO treatment to further boost the enzymatic degradation of soybean spent flakes is explored.

Research goals
As described in Paper 1 and Chapter 7, the cellulosic content of the soybean polysaccharides contributes significantly to the water insolubility of soybean spent flakes. Prior studies utilizing chemical extraction (detailed in Chapter 4) or selective enzymatic degradation (detailed in Chapter 6) focused exclusively on the pectin and hemicellulose polysaccharides of soybean. However, the linkage analysis and solubilization approaches presented in Paper 1 have highlighted the importance of the cellulose in soybean spent flakes. As a result of this finding, investigations into the enzymatic degradation of the cellulosic components of soybean were conducted.

As described in Subsection 6.1.2, soybean cellulose has shown resistance to degradation with traditional glycoside hydrolases (GHs). For that reason, a goal of the research presented in Paper 2 was to test the hypothesis that pretreatment methods could be utilized to improve the enzymatic digestibility of soybean spent flakes. In addition, a central hypothesis of this thesis has been

that knowledge of the structural elements of soybean polysaccharides can unlock new approaches for modification and/or degradation of this material. Utilizing knowledge of the cellulosic content of soybean from Paper 1 along with knowledge of the resistance of this material to traditional enzymatic degradation, LPMOs were evaluated for their activity on soybean spent flakes. As described in Chapter 7, LPMOs have shown the ability to enzymatically degrade cellulosic regions that are highly resistant to degradation with more traditional enzymes. This investigation of LPMO activity was used to test the hypotheses that enzymatic degradation can be used to solubilize soybean polysaccharides. In addition, LPMO treatments of soybean spent flakes allowed for testing of the hypothesis that LPMOs can be utilized to boost GH degradation of soybean cellulose.

Main results

*Tr*Cel61A activity was investigated on phosphoric acid swollen cellulose (PASC), Avicel® microcrystalline cellulose, galactan, cellopentaose, and cellohexaose. Of these five substrates, *Tr*Cel61A showed significant activity only on PASC. Using HPAEC-PAD, MALDI-TOF, and ESI-MS, the oxidation products were characterized to show C1 oxidation, C4 oxidation, and doubly oxidized species (Figure 1 in Paper 2). For this reason, *Tr*Cel61A was classified as a type-3 LPMO (Vu et al., 2014). A notable finding was the relatively large abundance of C4 oxidized products in comparison to previous work by Tanghe et al. with this enzyme (2015). The observation that LPMO activity on soybean cellulose shows strong C4 oxidation signals was also seen during the research for Paper 3 and is discussed in more detail in Chapter 9. During this preliminary substrate screening, *Tr*Cel61A did show some minor activity on Avicel®. However, this could not be verified with MALDI-TOF, and the relative abundance of oxidized products was extremely low. Overall, these results identified *Tr*Cel61A as a type 3 LPMO that is active on amorphous cellulose and inactive on soluble oligomers and crystalline cellulose.

Investigations into *Tr*Cel61A activity on soybean spent flakes showed no detectable oxidation, despite efforts to significantly increase both enzyme dosage and reaction time. Due to the observed lack of enzyme activity on Avicel®, it was hypothesized that the cellulose in soybean exists in a crystalline state that *Tr*Cel61A cannot degrade. For this reason, NaOH pretreatment of the soybean polysaccharides was investigated (further detailed in Paper 2). As described in Subsection 7.2.1, the purpose of this treatment is twofold: to convert the cellulose into the cellulose II allomorph for improved enzymatic digestibility and to remove hemicellulose and pectin polysaccharides that may sterically hinder the ability of *Tr*Cel61A to act on the soybean cellulose. These efforts were successful as demonstrated in Figure 7.3 from Chapter 7 and Figure 3 from Paper 2. Following pretreatment, *Tr*Cel61A showed significant activity on soybean spent flakes (Figure 4 from Paper 2). MS-MS fragmentation showed strong evidence of C4 oxidation, but no evidence of doubly oxidized products (C1 and C4 oxidation) could be detected.

*Tr*Cel61A activity alone did not show the ability to solubilize a significant portion of the soybean polysaccharides. However, *Tr*Cel61A treatment did show the ability to boost the glucose release from endo-β-1,4-glucanase treatment by up to 27% (Table 1 in Paper 2). This hydrolytic boosting effect was absent without the presence of ascorbic acid, indicating that the oxidative activity of the LPMO is indeed responsible for this effect.

Conclusion

The data presented in Paper 2 confirm Hypothesis (c) from Section 1.2, that pretreatment methods can be used to increase the enzyme accessibility of soybean polysaccharides. The results show a complete lack of LPMO activity on native soybean spent flakes and a significant level of activity following pretreatment. While only NaOH pretreatment was investigated in this work, this method demonstrates the utility of pretreatments to modify the composition and structure of soybean polysaccharides for enzymatic degradation. The final results from Paper 2 are unable to clarify if this effect is the result of the removal of substrate pectin and hemicellulose or due to changes in the crystalline structure of the cellulose. However, this is further explored in Paper 3, where additional LPMOs have been screened against the native and pretreated substrates. In addition, Hypothesis (d) from Section 1.2 was also confirmed with the demonstration of LPMO activity on soybean spent flakes. This oxidative activity was shown to significantly boost the activity of endo-β-1,4-glucanase on soybean cellulose, an effect which has been previously observed for other LPMOs on differing substrates (discussed in Subsection 7.1.4).

A goal of the research presented in Paper 2 was to investigate the hypothesis that targeted enzymatic degradation can assist in solubilizing soybean polysaccharides (Hypothesis (3) from Section 1.2). The results do confirm the ability of LPMO treatment to increase the generation of soluble cello-oligomers with endo-β-1,4-glucanase. However, it should be noted that LPMO treatment alone did not show any ability to solubilize the soybean polysaccharides. More notably, a significant solubilization of the remaining pectin and hemicellulose polysaccharides was not observed following the enzymatic degradation of pretreated soybean spent flakes with endo-β-1,4-glucanase. This either suggests that the insolubility of the non-cellulosic polysaccharide classes is not exclusively linked to the cellulose insolubility in the substrate, or that the enzymatic degradation employed in this research was not sufficient to fully degrade the cellulosic polysaccharides. The results suggest that the latter is the likely explanation, as only 34% of the substrate glucose was released with the enzymatic treatments described in Paper 2. Despite material pretreatment and hydrolytic boosting with LPMOs, it appears that portions of the soybean cellulose are still resistant to enzymatic degradation. In total, the results presented in Paper 2 confirm Hypothesis (2) from Section 1.2 by demonstrating how the structural data obtained in Paper 1 can be applied for the modification of soybean spent flakes. These hypotheses are further investigation in Paper 3,

where twenty-three additional LPMOs are evaluated for their activity on soybean polysaccharides.

9. Paper 3: A comparative study on the activity of fungal lytic polysaccharide monooxygenases for the depolymerization of cellulose in soybean spent flakes

Pierce, B. C.; Agger, J. W.; Zhang, Z.; Wichmann, J.; & Meyer, A. S. (2017). Carbohydrate Research, 449, 85-94.

This chapter contains a discussion of Paper 3 and its role in the work presented in this thesis. The full text of this paper can be found in the Publications section at the end of this thesis.

Brief summary
Paper 3 builds upon the findings of *Tr*Cel61A activity on soybean spent flakes from Paper 2. Twenty-three additional LPMOs from seven fungal organisms were expressed in *Trichoderma reesei*, and their N-terminal amino acids were sequenced. Each LPMO was evaluated for activity on PASC, Avicel®, native soybean spent flakes, and NaOH pretreated soybean spent flakes. From this survey, the LPMOs active on soybean spent flakes were evaluated with HPAEC-PAD and MS, and the top performing LPMOs were chosen for further characterization. The oxidation patterns for these LPMOS were evaluated, and their ability to boost the activity of GH release of glucose from soybean polysaccharides was investigated. In addition, the ability of LPMO combinations to release additional glucose was examined.

Research goals
The primary goal of the research in Paper 3 was to investigate the universal ability of LPMOs to oxidatively degrade the cellulose in soybean polysaccharides. While the results from Paper 2 demonstrated significant LPMO activity by *Tr*Cel61A on NaOH pretreated soybean spent flakes, the question remained whether this was a unique feature of *Tr*Cel61A or a more generalized LPMO effect. It was hypothesized that soybean polysaccharide activity was not a common trait for all LPMOs, and the research of Paper 3 aimed to test this hypothesis. In particular, a wide variety of LPMOs were examined in order to find commonalities between LPMOs active on soybean cellulose. The N-terminal sequences of these LPMOs were determined, with a goal of understanding possible connections between N-terminal histidine methylation and regioselectivity (as discussed in Subsection 7.1.3).

In addition, in Paper 2 *Tr*Cel61A only showed activity on amorphous cellulose (PASC) and pretreated soybean spent flakes. These results suggested a link

between a lack of activity on crystalline cellulose (Avicel®) and native soybean spent flakes. It was hypothesized that the crystallinity of the cellulose in native soybean could act as a limiting factor for LPMO activity. For this reason, a goal of the research presented in Paper 3 was to further investigate this hypothesis and determine if LPMO activity on the non-pretreated substrate is possible. Furthermore, combinations of LPMOs were investigated for their ability to provide synergistic effects, as it was hypothesized that multiple LPMOs showing differing oxidative activities could be used in combination to improve the enzymatic degradation of the substrate. In general, Paper 3 continues to explore a main hypothesis from this thesis that targeted enzymatic treatment of soybean polysaccharides can be used to solubilize the soybean spent flakes.

Main results

LPMOs were screened from the following fungal sources, with the number from each source indicated in parenthesis: *Trichoderma reesei* (1), *Chaetosartorya cremea* (2), *Aspergillus fumigatus* (5), *Aspergillus terreus* (8), *Myceliophthora thermophila* (3), *Penicillium citrinum* (2), *Malbranchea cinamomea* (2), and *Talaromyces leycettanus* (1). N-terminal sequencing of these proteins showed that all were expressed with an N-terminal histidine. As described in Subsection 7.1.3, this N-terminal histidine is associated with LPMO oxidative activity due to its presence in the active site of the enzyme. In addition, all proteins showed the presence of N-terminal histidine methylation; however, four LPMOs showed a higher abundance of non-methylated N-terminal ends. The LPMOS were evaluated for their activity on PASC, Avicel®, native spent flakes, and NaOH pretreated spent flakes (results shown in Paper 3 Table 2). The majority of the LPMOs screened showed activity on the amorphous cellulose of PASC, with three showing no activity. These three enzymes also displayed no activity on any substrates from this screening, despite confirmation that the sequences were correctly expressed. In addition, seven LPMOs demonstrated oxidative degradation of Avicel®, something which was not observed for *Tr*Cel61A in Papers 2 and 3. Interestingly, six of the seven LPMOs showing activity on Avicel® showed oxidative activity on NaOH pretreated soybean spent flakes, with *Tr*Cel61A being the only LPMO to show activity on pretreated soybean spent flakes but not Avicel®. Of particular note is the observation that the LPMOs showing the strongest activity on Avicel® also showed the best performance on pretreated spent flakes. Despite screening a selection of LPMOs, none of the investigated enzymes were capable of oxidative degradation of the native soybean spent flakes. These results, in combination with the observed activity on Avicel®, suggest that the hemicellulose and pectin content of the soybean polysaccharides plays a larger role than the cellulose crystallinity in determining LPMO activity.

The three LPMOs showing the best activity on the NaOH pretreated spent flakes were selected for further screening (*Tr*Cel61A, Aspte6, and Talle1). Figure 1 from Paper 3 shows the observed ESI MS-MS fragmentation patterns for the DP2 oxidized products from treatment of PASC with these LPMOs. These results show fragmentation products which are highly indicative of C4

oxidation (further described in Subsection 7.3.1). In addition, and as described in Paper 3, HPAEC-PAD profiles following beta-glucosidase treatment also showed strong evidence of C4 oxidation in samples of NaOH pretreated spent flakes treated with these enzymes. The three top-performing LPMOs were evaluated for their ability to boost the glucose release by endo-β-1,4-glucanase and beta-glucosidase from 0-72 hours. Each of these LPMOs - *Tr*Cel61A, Aspte6, and Talle1 - showed the ability to increase the glucose release from this enzymatic treatment by 55-88%. These results are comparable to those obtained for the hydrolytic boosting observed with *Tr*Cel61A in Paper 2. Finally, combinations of the top performing enzymes were evaluated; however, no significant increases in glucose release could be observed in these studies.

Conclusion

The results presented in Paper 3 confirm Hypothesis (e) from Section 1.2, showing selectivity by certain LPMOs for activity on soybean polysaccharides. Examining the amino acid sequences, presence of N-terminal methylations, and oxidation patterns of the LPMOs active on this substrate, a connection between C4 oxidative activity and soybean polysaccharide activity is observed. This connection appears to be substrate mediated, as *Tr*Cel61A showed good evidence of C1 oxidation for PASC but a strong preference for C4 oxidation on soybean polysaccharides. These results confirm Hypothesis (f) from Section 1.2; however, connections to protein sequence traits and/or N-terminal methylation could not be identified. Adding to these results, a connection between Avicel® activity and NaOH pretreated soybean polysaccharide activity was observed. These results, combined with the lack of observed activity on native spent flakes, indicate commonalities between the LPMOs which show activity on soybean polysaccharides. For activity to be observed, the substrate must first be enriched in cellulosic content through hemicellulose removal. In addition, the enzyme will most likely show activity on crystalline cellulose. From the analysis presented in Paper 3, it appears that the primary role of the NaOH pretreatment is in hemicellulose and pectin removal, and not in the conversion of cellulose allomorphs as described in Subsection 7.2.1. This is confirmed by the lack of any observed activity on the native soybean spent flakes, which still contain the significant hemicellulose and pectin branching described in Section 3.3 and Paper 1.

The hydrolytic boosting results presented in Paper 3 indicate that this is a generalized effect which may be performed by differing LPMOs, as long as they show strong oxidative activity on the substrate. This is illustrated by Figure 3 in Paper 3, where the top-performing LPMOs (*Tr*Cel61A, Aspte6, and Talle1) all show similar glucose release. In addition, investigations into LPMO combinations for improved enzymatic degradation did not show any effect. This is most likely due to the strong similarities between all soybean-polysaccharide-active LPMOs, most notably their propensity for C4 oxidative degradation. The similarity in degradation pattern results in nearly identical levels of

glucose release when a portion of the enzyme dosage is replaced with another, similarly active, LPMO (Figure 4 of Paper 3). In total, the results presented in Paper 3 confirm Hypothesis (3) from Section 1.2, showing the utility of targeted enzymatic treatment of soybean polysaccharides to solubilize this recalcitrant material.

10. Conclusion

The overall aim of this work has been to explore the structure and enzymatic degradation of soybean polysaccharides, with the future goal of utilizing this knowledge to develop more valuable products from this abundant and underutilized material. The strategy employed was two-fold, initially focusing on the structural characterization of the material through linkage and compositional analysis, and later focusing on chemical and enzymatic degradation for material solubilization. As discussed in this thesis, a primary drawback of soybean polysaccharides is their inherent lack of water solubility, a property that significantly limits their use in food applications. For this reason, a goal of the thesis was to further understand the compositional reasons for this insolubility and explore approaches to improve upon it.

The first major hypothesis of this work, namely that soybean polysaccharides are structurally unique and that their insolubility is defined by the structural make-up of the carbohydrates, was explored in all three publications presented here. Paper 1 describes the first reported linkage analysis data of the whole soybean polysaccharide fraction, instead of only partial linkage data from chemically extracted fractions. The results from this work highlight the complexity of the polysaccharide classes in soybean cell walls and, in general, confirm previous reports of the composition of soybean polysaccharides. In addition, the results describe the unique structural features of soybean polysaccharides, such as the extensive arabinan/arabinogalactan branching of the pectin components. This observed branching gives a pectin fraction that contains significantly more arabinose and galactose than galacturonic acid, and is therefore quite unique when compared to more traditional pectin from citrus peel, apple, or sugar beet (Andersson et al., 2006). These findings are in accord with previously published data on soybean polysaccharides (Li, Lu, et al., 2012). Interestingly, past research has demonstrated that higher amounts of neutral sidechains in pectin increase the observed binding of pectin and cellulose in the cell wall (Zykwinska et al., 2005). The data presented in Paper 1 also highlights the compositional consistency of soybean spent flakes, demonstrating remarkable stability in carbohydrate, protein, fat, and mineral levels over a period of almost two years.

More importantly, Paper 1 provides the first reported quantification of soybean polysaccharide composition, which previously could only be indirectly and inaccurately inferred from the yields and compositional information of chemical extracts. A notable finding from this work was the significant cellulose content in soybean (23.5%) and its recalcitrance to chemical solubilization with hydrogen peroxide treatment. The only previously reported quantification of cellulose in soybean spent flakes found only 12% cellulose (Carter et al., 1978); however, this analysis was performed with a simple colorimetric assay and is not congruous with the higher reported glucose levels of more recent studies

(Li, Lu, et al., 2012). Overall, the quantification reported in this work is a significant step forward for the understanding of this material and for the development of approaches for its utilization.

In addition, the peroxide treatment reported in Paper 1 demonstrated that the majority (>70%) of soybean polysaccharides can be solubilized in high molar mass, creating a soluble fraction enriched in the pectin and hemicellulose polysaccharides of the raw material. Coupled with knowledge of the resistance of soybean cellulose to chemical extraction and its insolubility following the acidic extraction in the SSPS process (Nakamura et al., 2000), these results suggest that the insolubility of soybean polysaccharides is primarily driven by the cellulosic content. This connection is notably absent from previous reports on the cell wall composition of soybean polysaccharides, as the primary aim of these studies was typically centered around pectin extraction. As such, the insolubility of the pectin fractions was primarily attributed to more traditional Ca^{2+} bridged cross-linking as described in Subsection 2.1.3 (Huisman et al., 1998; Yamaguchi, Ota, et al., 1996).

Due to the observed connection between material insolubility and cellulose content, Papers 2 and 3 investigated approaches for improving the enzymatic degradation of the soybean cellulose fraction using LPMOs. These enzymatic treatments demonstrated the ability of LPMOs to boost the enzymatic degradation of pretreated soybean cellulose by traditional glycoside hydrolases (GHs), an effect that has been previously reported for other substrates (Hemsworth et al., 2015). The data obtained in this thesis represents the first reported evidence of both LPMO activity and hydrolytic boosting of a GH5 endo-β-1,4-glucanase on soybean cellulose. An important finding of this work was the necessity of material pretreatment in order for LPMO activity to be observed. The results of Paper 3 suggest that the primary role of this pretreatment is to remove hemicellulose and pectin components, subsequently enriching the substrate in cellulose and increasing enzyme accessibility. It is interesting to note that the limited cellulose degradation (~35%) observed in LPMO and GH treatments did not result in the release of soluble pectin or hemicellulose polysaccharides. This either indicates that the portions of these fractions that are amenable to solubilization were previously removed in the material pretreatment, or that the portions of the cellulose primarily involved in interactions with the non-cellulosic polysaccharides are not affected by this enzymatic treatment. In total, the results confirm Hypothesis (1) from this thesis; however, it is apparent that future work is required to understand the precise interactions between the non-cellulosic polysaccharides and the cellulosic polysaccharides that serve to limit the solubility of the material.

The second hypothesis of this thesis, that structural elucidation of soybean polysaccharides will unlock new opportunities for modification of the material, was also demonstrated in the three publications presented here. While the development and testing of materials for food applications was outside the

scope of this thesis work, an important goal of this research was to demonstrate how structural information could be utilized in the development of modification and solubilization approaches for soybean polysaccharides. This was done with the intention that the approaches presented in this thesis, or alternative approaches developed from the structural information presented here, could later assist in the development of expanded applications for this substrate. As described in Chapters 4 and 6, the quantification of soybean polysaccharide composition presented in Paper 1 allows for the development of customized chemical and enzymatic degradation approaches. The peroxide solubilization process presented in this thesis was developed based on this information, and the results of Paper 1 highlight how structural information can be utilized to alter the solubilization process and enrich the resulting products in certain polysaccharide classes. For example, the peroxide solubilization process was adapted to result in a high molar mass, water-soluble fraction containing only 1.3% cellulose and 11.1% rhamnogalacturonan, compared with the 23.5% cellulose and 6.2% rhamnogalacturonan of the native spent flakes. In addition, the polysaccharide composition and physical properties of the resulting soluble material, combined with the knowledge of current food applications for soybean polysaccharides presented in Section 3.4, suggests opportunities in food functionality areas such as fiber fortification, beverage stabilization, emulsification, and dispersion (Williams & Phillips, 2009).

As described above, the enzymatic oxidative degradation explored in Papers 2 and 3 was driven by observations of the connection between material solubility and polysaccharide composition. The primary goal of these investigations was to understand this relationship through enzymatic degradation of the soybean cellulose. An important result from this work was the observation that only select LPMOs are capable of showing activity on pretreated soybean spent flakes, and that these LPMOs all share similar oxidative degradation profiles. More specifically, the vast majority of soybean polysaccharide-active LPMOs display strong evidence of C4 oxidation, as well as the ability to oxidatively degrade crystalline cellulose. In addition, structural studies of the effect of NaOH pretreatment in Papers 2 and 3 were able to identify a connection between enzyme accessibility and the polysaccharide content in this material, as described above. The work presented here confirms Hypothesis (2) from this thesis, and demonstrates the advantages of a structure-based targeted approach for the development of modification procedures.

The third hypothesis of this thesis, that targeted chemical and/or enzymatic treatment can help to solubilize soybean polysaccharides, was also a primary focal point for all three publications. As has already been described, chemical solubilization was demonstrated by the peroxide process presented in Paper 1. The most important result from the development of this process was evidence that the chemical treatment could be specifically tailored to adjust the resulting properties of the solubilized fraction. The high-molar mass, water-soluble product generated with this process is produced in high yields and

opens the possibility for new applications (e.g. fiber fortification, beverage stabilization, emulsification, and dispersion) using soybean polysaccharides, as described above. In addition, enzymatic approaches for solubilization were investigated in Papers 2 and 3. This work demonstrated the first evidence of LPMO activity on pretreated soybean polysaccharides and demonstrated the ability of these enzymes to boost the activity of traditional GHs on this substrate. These results confirm Hypothesis (3) and demonstrate that solubilization approaches can be utilized to overcome the significant limitations caused by the raw material's inherent lack of solubility.

Overall, the results presented in this thesis detail the structure of this complex material, and present novel chemical and enzymatic approaches for its degradation and solubilization. While future work is required to apply these findings for industrially relevant products, these results highlight the strength of structure-based modification approaches for soybean polysaccharides and open up a variety of new opportunities for utilization of this substrate in the future.

11. Perspectives

The research presented in this thesis has demonstrated the structural complexity of soybean polysaccharides and has proposed opportunities for the modification of this material, both chemically and enzymatically. Despite the questions answered, this work has also uncovered new challenges and opportunities to be addressed by future research in this area. The aim of this chapter is to discuss these issues, propose opportunities for future work, and discuss the potential impact of the results that have been presented in this thesis.

As described in Chapter 10, much of the work of this thesis has involved research to better understand the inherent insolubility of soybean polysaccharides and to develop approaches to improve upon this property. A key result of this work has been an understanding of the importance of the cellulosic polysaccharide fraction of soybean and the role it plays in the material's insolubility. However, fundamental questions still remain regarding how the cellulosic and non-cellulosic polysaccharides interact in the material. The research presented here suggests that disruptions of these interactions could significantly increase the solubility of soybean polysaccharides. However, the limited efficacy of cellulase digestion, even in the presence of LPMOs which increase degradation through hydrolytic boosting, indicates that there is still more to understand about how to disrupt these interactions. While the chemical extractions described in Chapter 4 offer some insight into these properties, these processes still only result in mixtures of polysaccharide classes at relatively low yields. In addition, another challenge encountered in this work was the difficulty in adapting NMR-based structural characterization techniques to the analysis of soybean polysaccharides. This is due to the heterogeneity of the material, with solubilized fractions typically containing a wide variety of polysaccharide classes and linkages. While basic information about the monosaccharide constituents can be discerned from these complex spectra, detailed structural characterization is extremely difficult. Approaches to address these challenges typically involve the separation and purification of more homogeneous carbohydrate fractions; however, challenges remain due to the lack of standards for these purified components. Nevertheless, NMR remains a powerful analytical tool for carbohydrate structural analysis, and future work may benefit from this technique if the appropriate purification steps are taken.

While the work presented here has focused exclusively on the polysaccharides in soybean, investigations into the residual protein which remains unextracted in the soy protein isolate process should be a focus of future work. Proteins which are entrapped in the insoluble cell wall fraction are present at appreciable levels (~10%) and could be viewed as lost yield from a protein production mindset. The SDS-PAGE gel presented in Subsection 3.2.2 demonstrates similarities and differences between these entrapped proteins

and those typically extracted in the soy protein isolate process. Going forward, it will be important to develop a deeper understanding of the polysaccharide and protein interactions in this material, in order to enable the development of processes to release additional protein and increase yields.

Papers 2 and 3 of this thesis present the first evidence of LPMO activity on soybean polysaccharides, and with this comes many opportunities for future work in this area. Currently, LPMO activity has only been observed following NaOH pretreatment of the soybean spent flakes. The evidence in Paper 3 suggests that the role of this pretreatment is primarily to remove non-cellulosic polysaccharides and improve enzymatic accessibility of the soybean cellulose. In an effort to bypass this pretreatment, preliminary experiments were performed involving enzymatic digestion with endo-β-1,4-galactanase in combination with LPMOs. The hypothesis behind these experiments was that removal of a significant amount of the galactan and arabinogalactan content could serve to improve the LPMO accessibility to the cellulose. However, the results of these experiments showed that the addition of galactanase does not result in any observable LPMO activity. Nevertheless, investigations into the enzymatic degradation of non-cellulosic components to increase accessibility to the cellulose components still represents a promising opportunity for future research.

In addition, future experiments involving the further enzymatic degradation of soybean polysaccharides with a combination of enzymes are recommended. As outlined in Chapter 6, a variety of enzymes exist that show activity on the linkages contained in this substrate, and combinations of enzymes could result in fractions with unique and interesting properties. Coupled with the cellulase and LPMO treatment described in Papers 2 and 3, opportunities exist for the enzymatic generation of soluble oligo- and polysaccharides. Recent research has also demonstrated the efficacy of catalase addition in LPMO-containing enzymatic digestion mixtures (Scott, Huang, Frickman, Halvorsen, & Johansen, 2016). LPMOs generate superoxide as an oxidation product, which is transformed into hydrogen peroxide and subsequently forms hydroxyl radicals (a process described in Paper 1). Catalase is capable of the conversion of hydrogen peroxide to water and oxygen, preventing unwanted radical inactivation of enzymes in the reaction. While the presence of excess substrate can effectively absorb these destructive products (Kittl, Kracher, Burgstaller, Haltrich, & Ludwig, 2012), catalase has been shown to limit unintended inactivation of enzymatic digestion mixtures and boost overall reaction yields (Scott et al., 2016). For these reasons, it is recommended that future research investigate the effects of catalase addition in hydrolytic boosting studies involving soybean polysaccharides and LPMOs.

Currently, the insolubility of soybean spent flakes limits this material's potential for use in food applications. The peroxide solubilized soybean polysaccharides generated in Paper 1 represent a unique material that has the potential

to greatly expand the applications of soybean spent flakes. The soluble product generated by this process is a large molar mass, water-soluble polysaccharide composed of primarily arabinogalactan, arabinoxylan, rhamnogalacturonan, and arabinan. In order to determine the true potential of this material, future work will need to focus on the testing of this material in applications such as fiber fortification, emulsification, dispersion, and beverage stabilization. In addition to the application of oxidizing enzymes to soybean polysaccharides, Paper 3 also serves to introduce a variety of LPMO proteins and opportunities exist for the application of these LPMOs in other diverse applications.

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Formation of water-soluble soybean polysaccharides from spent flakes by hydrogen peroxide treatment

Brian C. Pierce[a,d,*], Jesper Wichmann[a], Tam H. Tran[b], Roshan Cheetamun[c], Antony Bacic[c], Anne S. Meyer[d]

[a] DuPont™ Nutrition Biosciences ApS, Edwin Rahrs Vej 38, Brabrand 8220, Denmark
[b] DuPont™ Protein Solutions, 4300 Duncan Avenue Saint Louis, MO 63110, USA
[c] ARC Centre of Excellence in Plant Cell Walls, School of Biosciences, University of Melbourne, Melbourne VIC, 3010, Australia
[d] Department of Chemical and Biochemical Engineering, Center for Bioprocess Engineering, Technical University of Denmark, Søltofts Plads, Building 229, Kgs. Lyngby 2800, Denmark

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ABSTRACT

In this paper we propose a novel chemical process for the generation of water-soluble polysaccharides from soy spent flake, a by-product of the soy food industry. This process entails treatment of spent flake with hydrogen peroxide at an elevated temperature, resulting in the release of more than 70% of the original insoluble material as high molar mass soluble polysaccharides. A design of experiment was used to quantify the effects of pH, reaction time, and hydrogen peroxide concentration on the reaction yield, average molar mass, and free monosaccharides generated. The resulting product is low in protein, fat, and minerals and contains predominantly water-soluble polysaccharides of high molar mass, including arabinan, type I arabinogalactan, homogalacturonan, xyloglucan, rhamnogalacturonan, and (glucurono)arabinoxylan. This treatment provides a straightforward approach for generation of soluble soy polysaccharides and opens a new range of opportunities for this abundant and underutilized material in future research and industrial applications.

1. Introduction

Soy polysaccharides are produced as an industrial by-product from the manufacture of soy milk, tofu, and soy protein isolate. When resulting from either soy milk or tofu processing they are found in the okara fraction and following soy protein isolate production they make up the majority of the residual soy spent flakes (Li, Lu, Nan, & Liu, 2012; Lusas, 2000). Both okara and soy spent flakes are produced in large quantities globally with approximately 8×10^5, 3.1×10^5, 2.8×10^6 tons of okara produced by the Japanese, Korean, and Chinese tofu industries each year, respectively (Zhong & Zhao, 2015). The majority of these materials are either sold into low value applications or treated as waste that must be disposed of in an environmentally sustainable manner (Li et al., 2013). Thus, it is of significant interest to further characterize soy polysaccharides and develop alternate higher-value applications. However, a significant challenge when working with soy polysaccharides is their limited water solubility (O'Toole, 1999). This property makes detailed analytical characterization difficult and has slowed the development of novel applications and products from this material. Because of this, a process for the generation of a water soluble, high molar mass polysaccharide material from soy is needed to improve future utilization.

Due to the material's limited solubility, much of the research on and novel product development with soy polysaccharides has involved either chemical extraction or solubilization of the polysaccharides prior to analysis. Early efforts to investigate the make-up of insoluble soy polysaccharides involved hot water extraction (Aspinall & Whyte, 1964; Morita, 1965) and chemical treatment with ammonium oxalate, ethylenediaminetetraacetic-cacid (EDTA), or alkali (Aspinall, Hunt, & Morrison, 1966). More recently, researchers have focused on acidic extraction (Nakamura,

Abbreviations: H_2O_2, hydrogen peroxide; HG, homogalacturonan; AG, arabinogalactan; XG, xyloglucan; HM, heteromannan; SEC-MALS, size-exclusion chromatography with multi-angle light scattering; GC–MS, gas-chromatography–mass spectrometry; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detection.

* Corresponding author at: DuPont™ Nutrition Biosciences ApS, Edwin Rahrs Vej 38, Brabrand 8220, Denmark. Fax: +45 8625 1077.
E-mail addresses: brian.pierce@dupont.com, bpierce72@gmail.com (B.C. Pierce), jesper.wichmann@dupont.com (J. Wichmann), tam.h.tran@dupont.com (T.H. Tran), roshanc@unimelb.edu.au (R. Cheetamun), abacic@unimelb.edu.au (A. Bacic), am@kt.dtu.dk (A.S. Meyer).

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Furuta, Maeda, Nagamatsu, & Yoshimoto, 2000; Nakamura, Furuta, Maeda, Nagamatsu, & Yoshimoto, 2000; Nakamura, Furuta, Maeda, Takao, & Nagamatsu, 2002) and the use of various chelating agents (Huisman et al., 2001; Li et al., 2012; Mateos-Aparicio, Redondo-Cuenca, & Villanueva-Suárez, 2010; Ouhida, Pérez, & Gasa, 2002) to selectively solubilize different polysaccharide types from the raw material. These approaches have been successful, although they often rely on multiple sequential extractions, only solubilize select polysaccharide types, or result in unwanted degradation of the material to monosaccharides.

The use of peroxides to solubilize recalcitrant materials has been demonstrated for many diverse polysaccharide-rich materials, such as barley straw (Sun, Sun, Fowler, & Tomkinson, 2002), chitosan (Chang, Tai, & Cheng, 2001; Xia, Wu, & Chen, 2013), corn fiber (Doner, Chau, Fishman, & Hicks, 1998; Doner & Hicks, 1997), and peach gum (Yao, Cao, Pan, & Wu, 2013). These treatments typically employ high levels (between 15–60% (w/w)) of peroxide based on dry substrate solids, with temperatures ranging from 25–80 °C. Another common application of peroxides is in the bleaching of materials to give a whiter appearance (Strukul, 1992). These treatments typically employ lower levels (between 1–5% (w/w)) of peroxide based on dry substrate solids and higher temperatures ranging from 90–110 °C (Hage & Lienke, 2006). In contrast to solubilization applications, the goal of bleaching applications is to retain the polymeric structure of the material while affecting color-causing compounds. In order to accomplish this goal, it is common for chelating agents to be added to the reaction to minimize iron activated peroxide radical formation, the so called Fenton Reaction (Lloyd, Hanna, & Mason, 1997).

Polysaccharides are capable of undergoing non-enzymatic oxidative scission by the action of radical reactive oxygen species (ROS), most notably the hydroxyl radical (•OH) (Lindsay & Fry, 2006). If the hydroxyl radical abstracts a proton from a carbon involved in a glycosidic bond, cleavage of the polysaccharide backbone takes place and a decrease in average molar mass is observed. This scission process is displayed in Fig. 1 for a glycosidic bond in a galactan polymer. If the hydroxyl radical abstracts a proton from a non-glycosidic bonded carbon, the resulting product can contain either a more labile ester bond or an oxo group (Miller & Fry, 2001). This type of degradation has been demonstrated for plant cell wall polysaccharides such as β-glucan (Mäkelä, Sontag-Strohm & Maina, 2015), as well as pectin and xyloglucan using both Fenton and peroxidase-generated hydroxyl radicals (Schweikert, Liszkay, & Schopfer, 2000).

Soy polysaccharides contain pectic components, such as homogalacturonan (HG), xylogalacturonan, and rhamnogalacturonan in addition to other polysaccharides, such as galactan, arabinan, arabinogalactan (AG), xylan, xyloglucan (XG), and cellulose (Li et al., 2012; Nakamura et al., 2001). Because hydrogen peroxide (H_2O_2) has proven effective for treatment of polysaccharides, we hypothesized that it may be an effective method for degradation of the polysaccharides in soy. This degradation would result in a reduction in average molar mass and, coupled with the potential for selective solubilization of the more labile polysaccharide types, could lead to increased water-solubility of the soy polysaccharides.

In this paper, we explore this hypothesis and describe an alternative approach for the solubilization of soy polysaccharides using low levels of H_2O_2 in the presence of heat. This method results in partial degradation of the polysaccharides in high-yield to generate a high average molar mass water-soluble soy polysaccharide fraction containing only low levels of free monosaccharides. These three criteria (yield, avg. molar mass, and free monosaccharide level) were chosen because they allow a direct assessment of the degree of scission taking place during the H_2O_2 treatment. The average molar mass is an important response parameter as it helps determine the functionality, and consequently the potential future applications, of the finished product. The solubilization process presented here now allows for more detailed characterization of the structure of soy polysaccharides with analytical techniques and will help drive the development of novel uses for this abundant material.

2. Materials and methods

2.1. Materials

Soy polysaccharides were obtained from DuPont™ Nutrition & Health—Protein Solutions (St. Louis, MO) as a by-product (spent flakes) of the soy protein isolate production process. The material was lyophilized and ground to pass a USA-Standard ASTM 40-mesh screen. Forty four (44) samples of a commercially produced soy polysaccharide product, FIBRIM® 2000, were collected from DuPont™ Protein Solutions (St. Louis, MO) from soy protein isolate production runs from February 2012–December 2013 from both Identity Preserved (IP) and Genetically Modified (GM) soybean varieties.

L-fucose (Fuc), L-rhamnose (Rha), D-arabinose (Ara), D-galactose (Gal), D-glucose (Glc), D-xylose (Xyl), D-glucuronic acid (GlcA), and D-galacturonic acid (GalA) were purchased from Sigma (St. Louis, MO). BioXtra anhydrous sodium hydroxide pellets, H_2O_2 (30%), and BioUltra lithium nitrate ($LiNO_3$) were purchased from Sigma (St. Louis, MO). Concentrated sulfuric acid (95–98% w/v; H_2SO_4) was purchased from VWR (Radnor, PA) and used to generate a 12 M H_2SO_4 stock solution. Concentrated sodium hydroxide (50%; NaOH) was purchased from Sigma (St. Louis, MO) and used to generate a 2 M NaOH stock solution. Concentrated hydrochloric acid (30%; HCl) was purchased from Merck Millipore (Darmstadt, Germany) and used to generate a 2 M HCl solution. Sodium azide (NaN_3) was purchased from Merck Performance Materials (Darmstadt, Germany).

2.2. Soy polysaccharide H_2O_2 solubilization

Soy polysaccharides were diluted with MilliQ water to a concentration of 3% dry-basis solids. H_2O_2 (30%) was added to reach a final target concentration (w/w %) of peroxide based on the mass of the dry soy polysaccharides. The pH of the samples was adjusted with NaOH and/or HCl to reach the target experimental pH (between 2.8 and 11.2). The samples were sealed in screw-top test tubes from VWR (Radnor, PA), vortexed, and autoclaved in a Tuttnauer 2840EL benchtop autoclave at 121 °C for varying reaction times. Upon completion the material was separated by centrifugation in a Heraeus™ Multifuge™ X3R centrifuge from Thermo Scientific (Waltham, MA) at 12,000 × *g* for 10 min. The soluble fraction was removed by pipet and filtered through a 0.45 μm Whatman™ Puradisc syringe filter to remove any residual insoluble material. The remaining insoluble fraction was re-diluted in MilliQ water to the original reaction volume and vortexed. The centrifugation, filtration and dilution described above were repeated twice to collect first and second wash soluble fractions, and a final twice-rinsed insoluble fraction.

2.3. Material characterization

2.3.1. Composition

The moisture content of dry samples was measured in duplicate using a SMART System5™ microwave moisture analyzer from the CEM Corporation (Matthews, NC). Moisture content of wet samples was measured by oven solids at 110 °C for 18 h. The dry weight was then measured and the solid percentage was calculated based on the starting mass. Protein content was determined by combustion and determination of the total nitrogen content using a LECO TruMac® 630 N analyzer (St. Joseph, MI) with a protein factor of

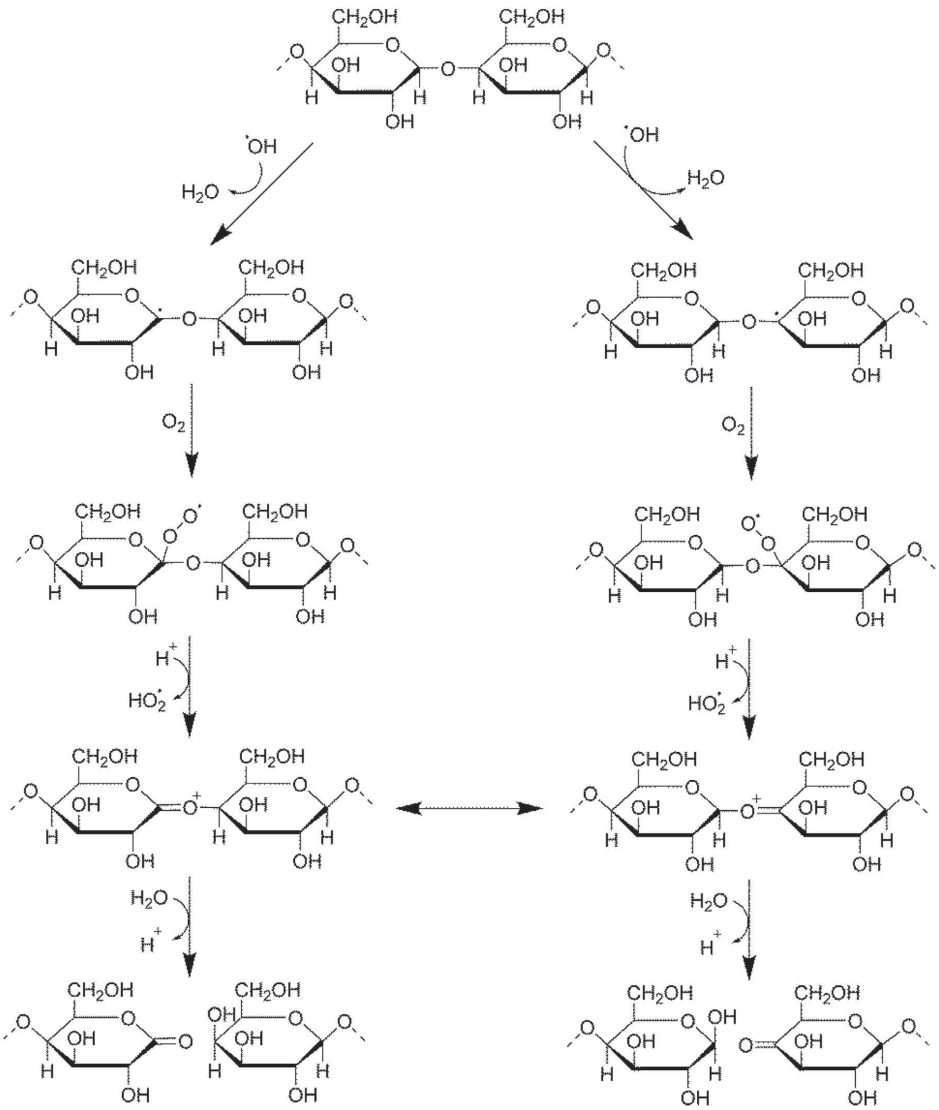

Fig. 1. Reaction mechanism for the hydroxyl radical (•OH) mediated scission of a glycosidic bond in a galactan polymer as proposed by Lindsay & Fry (2006). The horizontal arrow indicates a tautomerisation reaction.

6.25. Total mineral levels were determined by ashing the sample at 600 °C in a muffle furnace. The remaining residue was quantified to determine percent ash. Fat levels were determined by acid hydrolysis using AOAC method 922.06 (AOAC International, 2012). Carbohydrate by difference was calculated as the difference between 100% and the sum of the protein, fat, and mineral percentages for each sample.

2.3.2. Yield calculation

The reaction yield (Y) was calculated by dry mass according to Eq. (1), where $m_{initial}$ is the initial soy spent flake mass in dry basis; $m_{soluble}$ is the dry basis solid content of the soluble fraction collected, and m_{wash1} and m_{wash2} are the dry basis solid contents of the soluble first and second washes, respectively. The values of $m_{soluble}$, m_{wash1}, and m_{wash2} were calculated by multiplying the collected weight of the corresponding fraction by the percent solids of the fraction.

Eq. (1): Yield calculation for the H_2O_2 solubilization process

$$Y(\%) = 100 \times \frac{m_{soluble} + m_{wash1} + m_{wash2}}{m_{initial}} \quad (1)$$

2.3.3. High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

The monosaccharides in each sample were quantified by HPAEC-PAD. Chromatographic analysis was performed on a Dionex ICS-3000 Ion Chromatography system with a CarboPac® PA100

guard column (50 × 4 mm) followed by a CarboPac® PA100 analytical column (250 × 4 mm) and a PAD, all from Dionex Corporation (Sunnyvale, CA). The flow rate used was 1 mL/min under the following elution profile, with MilliQ water as eluent A and 600 mM NaOH as eluent B: 0–26 min, 1.5% B; 26–27.6 min, 1.5–30% B; 27.5–30 min, 30% B; 30–33.5 min, 30–75% B; 33.5–43.5 min, 75% B; 43.5–43.6 min, 75–1.5% B; 43.5–57.5 min, 1.5% B. Monosaccharides were quantified using external calibration derived from six standards containing a mixture of Fuc, Rha, Ara, Gal, Glc, Xyl, GlcA, and GalA. The concentration levels used for all monosaccharides ranged from 0.5–20 ppm (parts per million). The standard curves for each sugar were fitted with a linear calibration curve without offset with the exception of Fuc where a second order polynomial calibration curve without offset was used.

2.3.4. Complete acid hydrolysis for monosaccharide profile

Sulfuric acid (12 M) was added to the dry sample at a ratio of 6 mL/g sample and the samples were immediately vortexed to coat the dry sample in acid. The samples were incubated for 60 min in a 30 °C water bath with vortex mixing every 15 min. After incubation, the acid concentration was diluted to 0.49 M through the addition of MilliQ water. The samples were covered loosely and autoclaved in a Tuttnauer 2840EL benchtop autoclave for 40 min at 123 °C. The samples were then diluted with MilliQ water to the required level for chromatographic analysis, filtered using 0.45 μm syringe filters, and analyzed by HPAEC-PAD as described in Section 2.3.3. A concentration for each monosaccharide was determined, converted to a molar concentration using the molecular weight of the corresponding sugar, and converted to molar percentage by dividing each individual result by the total. All monosaccharide determination experiments were performed in triplicate.

2.3.5. Free monosaccharide determination

H_2O_2-treated soluble fractions and samples which underwent complete acid hydrolysis (Section 2.3.4) were diluted, filtered using 0.45 μm syringe filters, and analyzed by HPAEC-PAD as described in Section 2.3.3. A concentration for each monosaccharide was determined, and this value was multiplied by the total sample weight to determine a mass of each sugar in the fraction. This mass was adjusted to account for the added weight from hydration of the monosaccharides by multiplying the values by the ratio of the unhydrated mass to hydrated mass for each monosaccharide (e.g. the Glc ratio would be 0.9 = 162.14/180.16). Finally, these values were divided by the starting dry basis weight of soy spent flake used to give a corresponding percentage of the starting material which was identified as free monosaccharides.

2.3.6. Size-exclusion chromatography with multi-angle light scattering (SEC-MALS)

The average molar mass (g/mol) of each sample was determined by SEC-MALS. Chromatographic analysis was performed on a system comprised of an UltiMate® 3000HPLC pump from Dionex Corporation (Sunnyvale, CA), a Gynkotek Gina 50 autosampler from Gynkotek Softron (Munich, Germany), a DAWN® EOS® MALS detector, and an Optilab® rEX refractive index (RI) detector from Wyatt Technology Corporation (Santa Barbara, CA). The columns used were sequentially linked and consisted of a SUPREMA Lux 10 μm guard column, SUPREMA Lux 100 Å 10 μm (8 × 300 mm) column, and two SUPREMA Lux 10 μm 3000 Å 10 μm (8 × 300 mm) columns from Polymer Standards Service GmbH (PSS) (Mainz, Germany). The eluent used was 0.5 M $LiNO_3$ with 200 ppm of N_3^-. This was created with 6.892 g of $LiNO_3$ and 0.624 g of NaN_3 dissolved in 2 L of MilliQ water and filtered through a 0.22 μm filter. Isocratic flow was used at a rate of 0.6 mL/min. Data was collected and analyzed using ASTRA® V software (version 5.3.4.20) from Wyatt Technology Corporation (Santa Barbara, CA). A refractive index increment (dn/dc) of 0.1465 was used for all soy polysaccharide samples. During analysis the column and detector temperatures were maintained at 40 °C. Molar-mass dispersity was calculated as the ratio of the mass-average molar mass ($\bar{M}_w$) to the number-average molar mass ($\bar{M}_n$) according to the 2009 IUPAC recommendations (Stepto, 2009).

2.3.7. Carbohydrate linkage determination by gas-chromatography–mass spectrometry (GC–MS)

The glycosidic linkages present in the generated samples were determined by GC–MS as outlined by Pettolino, Walsh, Fincher, & Bacic (2012). Samples underwent cell wall alcohol insoluble residue (AIR) preparation, α-amylase digestion for starch removal, carboxyl reduction, methylation, hydrolysis with trifluoroacetic acid (TFA), reduction, acetylation, and GC–MS determination. Results are reported in molar percentages as averages of duplicate analysis.

2.4. Design of Experiment (DOE)

DOE design was done using Minitab® 17 from Minitab Inc. (State College, PA). A central composite design of three factors: reaction time (min), reaction pH, and H_2O_2 concentration (w/w %) was used. A two-level full factorial design was conducted with all samples performed in duplicate, resulting in a total of 40 individual reactions. Reaction time ranged from 6.5–73.5 min, reaction pH ranged from 2.8–11.2, and H_2O_2 concentration ranged from 0.6–12.4%. For each reaction, the corresponding soluble yield, free monosaccharide level, and average molar mass were determined. Response surface equations with full quadratic terms and backward elimination of insignificant terms at a threshold of $\alpha = 0.1$ were generated for each response individually. This technique fits a second-order prediction equation to the data, where quadratic terms model the curvature observed in the experimental responses. This model was chosen due to the continuous nature of the response variables and the desire to optimize these responses based on the process variables (Khuri & Mukhopadhyay, 2010). The response variables of average molar mass and free monosaccharide percentage were transformed with a base-10 logarithm in order to achieve a normal distribution. In addition, a multivariate principal component analysis (PCA) was conducted with a correlation matrix to deconvolute the data and demonstrate the relationship between all response variables (Jolliffe, 2002).

3. Results and discussion

3.1. Soy polysaccharide baseline survey

Forty four (44) commercial FIBRIM® 2000 samples were analyzed for their protein, fat, mineral, and carbohydrate by difference composition, and samples were compared based on type (IP or GM). The target was to identify a single sample that could be used for subsequent testing and would be representative of an average soy polysaccharide spent flake material. No statistical difference between the GM and IP samples in either the compositional categories (Table 1) or in the monomeric sugar composition (Fig. 2) was observed. A representative spent flake sample was selected for further analysis based on this baseline evaluation (Table 1).

The sample selected was an IP soy polysaccharide sample collected from a DuPont™ Protein Solutions soy protein isolate process. The composition of the selected sample fell within the standard deviations of all parameters, with the exception of dry basis minerals (Table 1). While the representative sample showed a statistically different mean for dry basis mineral level when compared to the IP and GM baseline samples, the sample was selected because of its higher total carbohydrate level. In addition, our data suggests that the majority of the spent flake mineral content remains selectively with the insoluble fraction during H_2O_2

Table 1
Composition of commercial FIBRIM® 2000 samples and the chosen representative sample. Standard deviations given in parentheses. Values are significantly different ($p < 0.05$) from values in the same column if followed by different letters (a–b).

Sample	Dry basis protein (%)	Dry basis fat (%)	Dry basis minerals (%)	Dry basis carbohydrate by difference (%)
FIBRIM® 2000 identity preserved (IP) samples [$n = 21$]	11.28[a] (±1.93)	0.85[a] (±0.16)	4.17[a] (±0.29)	83.69[a] (±1.85)
FIBRIM® 2000 genetically modified (GM) samples [$n = 23$]	10.40[a] (±1.83)	0.94[a] (±0.18)	4.36[a] (±0.21)	84.30[a] (±1.80)
Selected representative soy spent flake sample	10.05[a]	0.82[a]	3.31[b]	85.82[a]

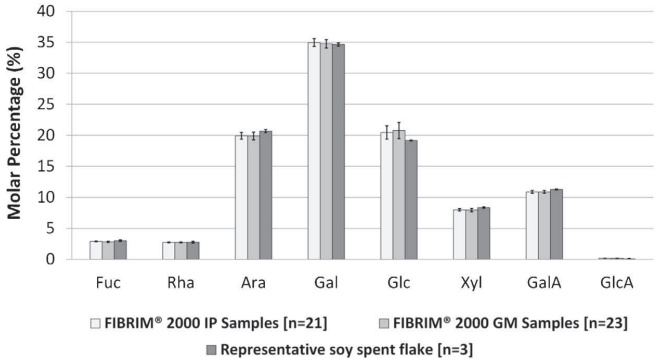

Fig. 2. Monomeric sugar composition of commercial FIBRIM® 2000 samples and the chosen representative sample. Data are shown as averages of duplicate analyses of each sample with standard deviations given by error bars. No significant differences between the levels of each monomer among the samples were observed ($p > 0.05$).

treatment, and therefore should not have a strong impact on the resulting soluble products. The samples showed a high degree of consistency in both proximate composition and sugar composition across the 23-month sampling period. The hydrolyzed sugar profile showed a typical sugar residue distribution for soy polysaccharides (Fig. 2), with the five most abundant monomeric units being Gal, Glc, Ara, GalA, and Xyl (Aspinall, 1988).

3.2. Soy polysaccharide solubilization

H_2O_2 treatment experiments were performed on the chosen representative soy polysaccharide sample with the goal of maximizing both the yield of water-soluble polysaccharides and the average molar mass of the resulting product, while minimizing the amount of free monosaccharides generated.

3.2.1. Effect of H_2O_2 concentration on solubilization at neutral pH

The effect of H_2O_2 concentration on the solubilization of soy polysaccharides was evaluated with a series of experiments performed at pH 7 and exposed to autoclave heating at 121 °C for 60 min (Fig. 3). These data demonstrate the ability of H_2O_2 to degrade the soy polysaccharides and create lower molar mass soluble fractions at high yields.

Soluble yields greater than 70% are observed, indicating excellent solubilization of the starting soy spent flakes. In addition, a decrease in yield is observed for H_2O_2 concentrations above 10% (w/w) dry basis fiber. The generation of monosaccharides shows a linear response to H_2O_2 concentration in the range of 0–30% H_2O_2 (R-squared of 0.993) (Fig. 3A). The average molar mass of soy polysaccharides treated without H_2O_2 corresponds to a degree of polymerization (DP) of roughly 5,000 (~800,000 g/mol), while the samples treated at 50% (w/w) H_2O_2 have an average molar mass corresponding to a DP of roughly 5 (~800 g/mol) (Fig. 3B). The SEC profiles for the H_2O_2 treated samples show a late eluting salt peak and a primary peak with molar-mass dispersity ranging from 2.99 (0% H_2O_2) to 1.87 (50% H_2O_2). As the H_2O_2 concentration increases this peak narrows and the molar-mass dispersity decreases, indicating that the molar mass distribution is becoming more uniform. The monomeric composition by acid hydrolysis for the H_2O_2 solubilized samples was also examined (data provided in Supplementary Fig. S3). For H_2O_2 concentrations from 0–10%, the monomeric composition remained relatively unchanged. However at increased H_2O_2 concentrations the monomer composition of the solubilized polysaccharides increased proportionally in Glc, Xyl, GalA, and GlcA and decreased in Ara and Gal. This trend demonstrates the varying susceptibility of both the glycosidic linkages and the sugar monomers to this treatment, with increasingly harsh reaction conditions capable of affecting the more recalcitrant polysaccharide types while causing degradation of the more labile monomers. Maximal yields were observed for H_2O_2 concentrations between 5 and 10% (w/w), and these levels corresponded to roughly DP 144 (~74,000 g/mol) and DP 34 (~5500 g/mol) polysaccharides, respectively (Fig. 3).

Based on these data, it is clear that H_2O_2 treatment causes degradation of the soy polysaccharides and results in a lower average molar mass. This most likely occurs via oxidative scission of the polysaccharides by hydroxyl radicals (Fig. 1) formed through transition metal mediated decomposition of H_2O_2 in the presence of trace iron levels in soy polysaccharides (Neyra, Paladino, & Le Borgne, 2015). In this reaction, iron(II) is oxidized by H_2O_2 to form iron(III), a hydroxide anion, and the hydroxyl radical. In the present study, the oxidation products of this degradation pathway were not directly identified and further studies are needed in order to fully verify and understand this degradation pathway in soy. To evaluate the impact of the processing conditions on this H_2O_2-induced degradation, a design of experiment was conducted based on the reaction parameters of pH, time, and H_2O_2 concentration.

3.2.2. Design of experiment (DOE) to model solubilization conditions

The goal of the DOE is to model the effects of pH, H_2O_2 concentration, and reaction time on the three chosen response variables of yield, log (monosaccharides), and log (avg. molar mass). This model allows an understanding of the interaction between the process variables, and enables optimization of the reaction conditions to fit

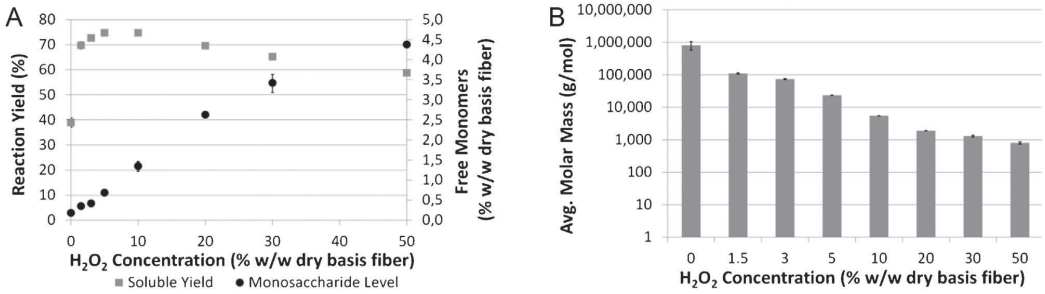

Fig. 3. (A) Soluble yield and free monosaccharide content vs. H_2O_2 concentration for solubilization reactions of 60 min at pH 7 and 121 °C. (B) Avg. molar mass (g/mol) on a logarithmic scale vs. H_2O_2 concentration for the products from A. All data are shown as averages of duplicate analyses with standard deviations given by the error bars. Representative SEC-MALS chromatograms are given in the Supplementary material (Supplementary Figs. S1 and S2).

Table 2
Response surface parameters for DOE models of yield, log (monosaccharides), and log (avg. molar mass). Squared terms for the three response factors included in the modeling and given in Supplementary Table S1, but omitted from this table for clarity.

	Term	Const.	Conc.	Time	pH	Conc.* Time	Conc.* pH	Time* pH
Yield (%)	Coded coefficient	61.05	3.18	10.280	−6.45	–	2.900	7.820
	Uncoded coefficient	120.40	−1.41	0.215	−18.06	–	0.332	0.156
	p-value	0.000	0.006	0.000	0.000	–	0.050	0.000
log (mono.)	Coded coefficient	−0.58	0.09	0.133	−0.37	–	–	–
	Uncoded coefficient	1.24	0.26	0.014	−0.54	–	–	–
	p-value	0.000	0.000	0.000	0.000	–	–	–
log (avg. molar mass)	Coded coefficient	5.51	−0.26	−0.294	0.50	−0.1729	–	0.173
	Uncoded coefficient	2.57	0.18	−0.009	0.68	−0.0025	–	0.003
	p-value	0.000	0.000	0.000	0.000	0.003	–	0.003

a response target (Khuri & Mukhopadhyay, 2010). The contributing factors, coefficients (both coded and uncoded), and p-values for the three response surface models are listed in Table 2. The coded coefficients are useful for direct comparisons between the different process variables, while the uncoded coefficients can be used in a response surface equation to predict the value of the corresponding response variable.

The reaction yields measured in the DOE ranged from 25.5–74.4%. The response surface fit for the reaction yield results in a standard error of regression (*S*) of 5.69, an *R*-squared of 85.73%, and an *R*-squared (predicted) of 76.46%. Comparison of the coded coefficients indicates that the reaction yield is optimized at lower pH and longer reaction times (Table 2). In addition, higher H_2O_2 concentrations result in an increased yield above pH 4.25, the point at which the negative contribution of the conc. term is offset by the quadratic conc.*pH term. These results suggest that the primary degradation mechanism below pH 4.25 is not peroxide mediated. It is likely that the primary degradation mechanism at these pH values is an acid hydrolysis nucleophilic substitution reaction.

The monosaccharide levels measured in the DOE ranged from 0.12–5.31% (w/w) of the starting soy polysaccharides. The response surface for the logarithm of monosaccharide level results in an *S* of 0.094, an *R*-squared of 94.69%, and an *R*-squared (predicted) of 91.00%. This model indicates that free monosaccharide levels are most dramatically influenced by the reaction pH, with increased levels occurring at lower reaction pH values. In addition, both increased reaction times and H_2O_2 levels result in a greater amount of free monosaccharides. The average molar masses measured in the DOE ranged from 3000–811,000 g/mol (DP 19–5000) with molar-mass dispersities ranging from 1.4–5.9, respectively. The response surface for the logarithm of average molar mass results in an *S* of 0.212, an *R*-squared of 91.29%, and an *R*-squared (predicted) of 83.38%. In general, we observe that decreased pH or longer reaction times result in lower average molar masses. At the same pH, increased H_2O_2 concentrations and reaction times also result in decreased average molar mass. For both of these models, these relationships are expected as they will result in increased degradation of the polysaccharides, subsequent release of monosaccharides, and a decrease in average molar mass from untargeted glyosidic bond cleavage.

These models demonstrate the relationship between a single response variable and our three chosen process variables. However, the primary interest is to understand how the response variables interact with each other. This was accomplished using a principal component analysis (PCA) where the response variables were represented by a smaller number of uncorrelated variables, called principal components. These components are calculated as a liner combination of the original variables, such that the first accounts for as much data variation as possible and the others have maximal variance while remaining uncorrelated to each other (Jolliffe, 2002). For this analysis, two principal components were able to account for 95.8% of the observed variance in the data. The first principal component (PC1) gave an eigenvalue of 2.35 and described 78.4% of the observed variance, while the second principal component (PC2) gave an eigenvalue of 0.52 and described 17.4% of the observed variance. The formulas for PC1 and PC2 are given in Equation 2, and the PCA biplot of the first two principal components is shown in Fig. 4.

Eq. (2). Calculation of principal component 1 (PC1) and principal component 2 (PC2) from yield, log(monosaccharides), and log(avg. molar mass)

$$PC1 = [0.513 \times \text{yield}] + [0.615 \times \log(\text{monosaccharides})] - [0.599 \times \log(\text{avg.molar mass})]$$

$$PC1 = [-0.853 \times \text{yield}] + [0.286 \times \log(\text{monosaccharides})] - [0.436 \times \log(\text{avg.molar mass})]$$

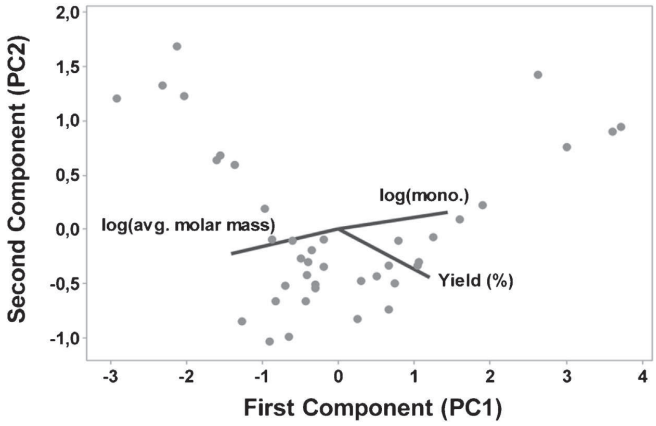

Fig. 4. PCA biplot of the first two principal components, where PC1 and PC2 are given by Equation 2.

Table 3
Composition of starting soy spent flakes and the H_2O_2 treatment products for the reaction at pH 7, 121 °C, 60 min, and 3% (w/w) H_2O_2.

Sample	Dry basis protein (%)	Dry basis fat (%)	Dry basis minerals (%)	Dry basis carbohydrate by difference (%)
Soy spent flake	10.05	0.82	3.31	85.82
H_2O_2 treatment: soluble fraction	6.81	0.38	0.99	91.82
H_2O_2 treatment: insoluble fraction	13.43	1.22	3.63	81.72

From Fig. 4 we observe an overall trend of an increase in the free monosaccharide level and decrease in the average molar mass as the yield increases. While our target conditions aim to maximize yield and average molar mass while minimizing free monosaccharides, we see that it is not possible to fully optimize all of these responses independently. It is therefore important to prioritize and weight the importance of these responses in order to determine the optimal conditions for each specific application. This is accomplished by simultaneously optimizing the three DOE response surface models (Table 2) with a weighted importance given to each of the three response variables. In this way, process variables can be selected to give an overall optimal set of conditions for multiple responses. For our purposes, H_2O_2 generated products were produced based on the DOE model with all response variables weighted equally, pH constrained to 7.0, and H_2O_2 concentration constrained to 3.0% (w/w dry basis fiber). These constraints were chosen to minimize H_2O_2 use and to operate at neutral pH for reaction equipment durability. The DOE model indicates an optimized reaction time of 60 min at 121 °C under these conditions. A reaction yield of 60% was achieved using these reaction conditions, 94% of the DOE model prediction of 64% yield.

3.2.3. *Compositional characterization of H_2O_2 solubilized soy polysaccharides*

Table 3 shows the protein, fat, minerals, and carbohydrate by difference for the products from the above optimized H_2O_2 treatment conditions. The soluble fraction from H_2O_2 treatment was enriched in carbohydrates and the resulting insoluble fraction contained a significant amount of the starting protein, fat, and minerals.

It is interesting to observe that the mineral and protein content of the starting soy spent flakes remained selectively with the insoluble fraction during this treatment, despite the expected water solubility of these components under these reaction conditions. This finding suggests an interaction (either covalent or non-covalent) between the polysaccharides, protein, and minerals in the insoluble fraction. In order to investigate the polysaccharide types in each of the resulting fractions, acid hydrolyzed monosaccharide profiles were obtained for each sample (Fig. 5).

From this analysis we observe that the H_2O_2 solubilized fraction is enriched in Ara and Gal when compared to the starting soy spent flakes. The insoluble fraction shows a corresponding decrease in these monosaccharides and an overall enrichment in Glc- and Xyl-containing polysaccharides. It is also interesting to note that the GalA content remains unchanged between the soluble and insoluble fractions, indicating a relatively equal compositional distribution between the two fractions. In addition, very low levels of GlcA were observed in the three fractions. In order to more-fully characterize these polysaccharides a complete glycosidic linkage profile was obtained by GC–MS (Table 4). These data align well with the monosaccharide compositions from HPAEC-PAD (Fig. 5), with differences most likely attributable to small differences in the sample preparation (e.g. TFA vs. sulfuric acid hydrolysis) between the two techniques. From these data we observe the presence of polysaccharide linkages associated with pectic, non-cellulosic, and cellulosic polysaccharide types.

Table 4 also lists the common polysaccharide classes associated with the various linkage types observed, and this can be used to assign the observed monosaccharide linkages into polysaccharide classes (see Table 5) as suggested by Pettolino et al. (2012).

Table 5 shows the calculated levels of various polysaccharide classes for these three samples using the assignment criteria suggested by Pettolino et al. (2012). Approximately 85% of the observed linkages were assigned to polysaccharide classes via this exercise and we observe that the H_2O_2 solubilization results in a final soluble product enriched in arabinan, type I AG, HG, rhamnogalacturonan, (glucurono) arabinoxylan, and XG polysaccharides. The assignment of 4-Glc requires additional explanation as this linkage can be found in cellulose, XG, HM, and starch (Table 4). As part of the linkage analysis the samples were treated with α-amylase to remove starch and the relative amount of 4-Glc assigned to XG and HM was equivalent to the amount of 4,6-Glc and 4-Man found in these polysaccharides, respectively. After assignment of any 4-Glc

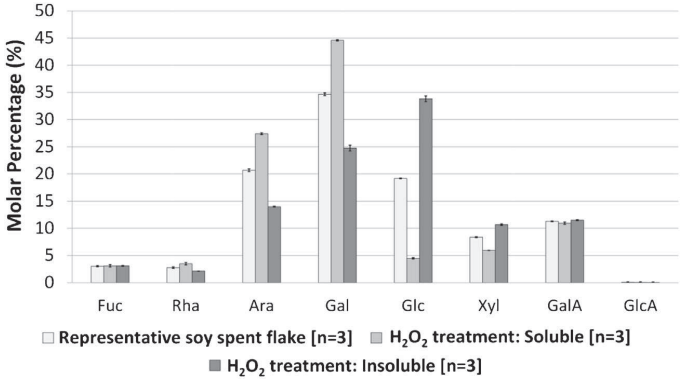

Fig. 5. Monosaccharide composition of the chosen representative soy spent flake sample and H_2O_2 treated samples at pH 7.0, 121 °C, 60 min, and 3.0% (w/w dry basis fiber) H_2O_2 addition. All measurements were performed in triplicate with standard deviations given by the error bars.

Table 4
Linkage (molar %) analyses of starting material and H_2O_2 solubilization products for the reaction at pH 7, 121 °C, 60 min, and 3% (w/w) H_2O_2. Values are averages of duplicate analysis and common polysaccharide types associated with each linkage type are shown.

Mono-saccharide	Linkage	Starting spent flake Mol%	H_2O_2 Treat. Soluble Mol%	H_2O_2 Treat. Insoluble Mol%	Common associated polysaccharide types
Ara(f)	Terminal	12.3	15.3	11.0	Type I/II AG, HX, XG, arabinan
	2-	0.4	0.1	0.5	Type I/II AG, HX, RGII
	3-	0.2	0.3	0.4	HX
	5-	2.1	2.5	2.5	Arabinan, Type II AG, HX
	3,5-	3.0	3.5	2.5	Arabinan
Ara(p)	Terminal	1.7	1.2	2.8	Type II AG
Gal(p)	Terminal	2.0	4.3	3.3	XG, Type II AG, HX, RGI, RGII
	4-	26.8	36.6	20.7	Type I AG/RGI
	3-	1.7	0.7	1.7	Type II AG
	6-	0.1	0.2	0.3	Type II AG, RGI, RGII
	3,4-	0.5	0.3	0.4	Type I AG
	2,4-	0.3	0.2	0.3	Type I AG
	3,4,6-	0.1	0.0	0.0	Type II AG
	2,3,4-	0.1	0.0	0.0	Type I AG
Glc(p)	Terminal	0.4	0.1	0.6	Across glucan classes
	4-	24.0	1.7	27.0	Cellulose, XG, HM, starch
	6-	0.2	0.1	0.2	–
	3,4-	0.3	0.9	0.4	–
	2,4-	0.2	0.0	0.2	–
	4,6-	0.5	0.4	0.4	XG, starch
	3,4,6-	0.1	0.0	0.0	–
Fuc(p)	Terminal	1.4	0.7	3.1	XG
Xyl(p)	2-	1.1	2.2	1.5	XG, RGII
	4-	9.8	12.4	8.4	HX
	2,4-	2.0	2.8	1.3	HX
	3,4-	0.2	0.0	0.2	HX
Rha(p)	Terminal	0.0	0.1	0.0	Type II AG, RGII
	2-	0.9	1.4	1.5	RGI
	2,4-	2.1	4.1	1.8	RGI
Man(p)	Terminal	0.2	0.2	0.0	–
	4,6-	0.0	0.0	2.4	HM
GalA(p)	Terminal	0.2	0.5	0.6	HG, RGI, RGII
	4-	4.7	6.5	3.7	HG, RGI
	3,4-	0.0	0.8	0.0	RGI
GlcA(p)	Terminal	0.1	0.0	0.3	HX, Type II AG, RGII

Abbreviations: (f)—furanose; (p)—pyranose; AG—arabinogalactan; HG—homogalacturonan; HM—heteromannan; HX—heteroxylan; RG—rhamnogalactan; XG—xyloglucan.

to XG and HM, the remaining 4-Glc identified is attributed to cellulose (Pettolino et al., 2012). Another important distinction is in the assignment of heteroxylans, a family of polysaccharides with a linear β-(1,4)-linked Xyl backbone and possible side chain substitutions of acetyl, GlcA, 4-*O*-methylglucuronic acid, and/or Ara. The presence and types of substitutions determine the classification of these polysaccharides as arabinoxylan (AX), glucuronoarabinoxylan (GAX), or glucuronoxylan (GX) (Bacic, Harris, & Stone, 1988; Faik, Jiang, & Held, 2014). The side chain composition is heavily material dependent, and precise classification of these compounds requires sequence determination. For the soy polysaccharides in this study, the presence of GlcA in the GC–MS and HPAEC-PAD analysis as well as observation of the expected Xyl branching for both Ara and GlcA substitution indicates the presence of glucuronoarabinoxylan. Due to the low levels of GlcA detected, this has been reported tentatively as (glucurono) arabinoxylan, with future work required to determine the exact composition of this polysaccharide in soy.

Table 5
Calculation of polysaccharide composition (mol%) of the starting spent flake and H_2O_2 solubilization products for the reaction at pH 7, 121 °C, 60 min, and 3% (w/w) H_2O_2, based on the data from Table 4 and assignment criteria from Pettolino et al. (2012).

Polysaccharide	Starting spent flake	H_2O_2 treated soluble	H_2O_2 treated insoluble
Arabinan	8.1	9.6	7.5
Type I AG	27.8	37.1	21.6
Type II AG	2.0	0.9	2.0
XG	2.7	3.4	2.5
Rhamnogalacturonan I/II	6.2	11.1	6.7
HG	1.6	2.2	0.3
(Glucurono) arabinoxylan	14.4	18.3	11.9
HM	0.0	0.0	4.7
Cellulose	23.5	1.3	26.7
Others (unassigned)	13.7	16.1	16.1

Abbreviations: AG—arabinogalactan; HG—homogalacturonan; HM—heteromannan; XG—xyloglucan.

The insoluble fraction was heavily enriched in cellulose (Robyt, 1998), demonstrating that this polysaccharide is resistant to solubilization in the H_2O_2 treatment conditions employed. This direct correlation between material solubility and cellulose content strongly suggests that the cellulosic components in the soy fractions are a major contributor to the overall soy spent flake insolubility. In addition, the compositional information in Table 3 indicates that these polysaccharides may be interacting (either covalently or non-covalently) with the residual protein and mineral components observed in the insoluble fraction. This protein-polysaccharide interaction is also demonstrated in the production of soy spent flakes, where residual protein remains with the insoluble polysaccharides even following multiple aqueous protein extractions. Overall these results correspond well to the various polysaccharide types previously identified in soy using chemical extraction techniques (Aspinall, 1988; Li et al., 2012), with one notable difference being the absence of 1,5-Xyl(f) in this analysis as previously reported by Li et al. (2012). In total, these linkage results provide a comprehensive overview of the polysaccharide classes present in soy spent flake and also clearly demonstrate the ability of the proposed H_2O_2 process to solubilize a significant majority of the pectic and non-cellulosic polysaccharides in soy.

4. Conclusions

The H_2O_2 process described above is a new process for generating water-soluble, high molar mass soy polysaccharides from soy spent flakes. In addition, the data indicates that the resulting properties of the polysaccharides can be tailored by manipulating the process conditions of pH, reaction temperature, and H_2O_2 concentration as described by the response surface models given in this publication. This process is able to create a soluble material enriched in both the pectic and non-cellulosic polysaccharides of soy, such as arabinan, type I AG, HG, rhamnogalacturonan, (glucurono) arabinoxylan, and XG. Further studies are needed in order to characterize the functionality and oxidation products of these H_2O_2 solubilized soy polysaccharides; however the polysaccharide composition, water solubility, and large average molar mass suggest that potential food applications may exist in fiber-fortification, emulsification, dispersion, and beverage stabilization (Williams & Phillips, 2009). This H_2O_2 treatment process for soy polysaccharides opens new possibilities for structural characterization of this material and will enable novel applications of this complex and underutilized resource in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2016.02.082.

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Oxidative cleavage and hydrolytic boosting of cellulose in soybean spent flakes by *Trichoderma reesei* Cel61A lytic polysaccharide monooxygenase

Brian C. Pierce[a,b,*], Jane Wittrup Agger[b], Jesper Wichmann[a], Anne S. Meyer[b]

[a] *DuPont™ Nutrition Biosciences ApS, Edwin Rahrs Vej 38, Brabrand, 8220, Denmark*
[b] *Department of Chemical and Biochemical Engineering, Center for Bioprocess Engineering, Technical University of Denmark, Søltofts Plads, Building 229, Kgs. Lyngby 2800, Denmark*

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ABSTRACT

The auxiliary activity family 9 (AA9) copper-dependent lytic polysaccharide monooxygenase (LPMO) from *Trichoderma reesei* (EG4; *Tr*Cel61A) was investigated for its ability to oxidize the complex polysaccharides from soybean. The substrate specificity of the enzyme was assessed against a variety of substrates, including both soy spent flake, a by-product of the soy food industry, and soy spent flake pretreated with sodium hydroxide. Products from enzymatic treatments were analyzed using mass spectrometry and high performance anion exchange chromatography. We demonstrate that *Tr*Cel61A is capable of oxidizing cellulose from both pretreated soy spent flake and phosphoric acid swollen cellulose, oxidizing at both the C1 and C4 positions. In addition, we show that the oxidative activity of *Tr*Cel61A displays a synergistic effect capable of boosting endoglucanase activity, and thereby substrate depolymerization of soy cellulose, by 27%.

1. Introduction

Soy polysaccharides are a carbohydrate-rich by-product resulting from the manufacture of soy milk, tofu, and soy protein isolate. They are composed predominantly of water-insoluble cell wall polysaccharides of high molar mass, with constituent polysaccharide types including type I arabinogalactan, cellulose, arabinoxylan, rhamnogalacturonan, arabinan, xyloglucan, and homogalacturonan [1]. This material is generally referred to as okara when resulting from soy milk or tofu production, and soy spent flake following soy protein isolate production [2]. Both of these products are produced in large quantities globally with approximately 2.8×10^6 tons of okara produced by the Chinese tofu industries each year [3] and 8×10^5 tons of okara discarded annually in Japan [4]. Currently the vast majority of this material is disposed of as waste at significant cost, utilized in low value applications such as fertilizer or animal feed, or burned [5,6]. For these reasons, there is significant interest in increasing the utilization of this material, and recent efforts with this aim have centered on the solubilization of the recalcitrant polysaccharides utilizing acidic extraction [7], enzymatic degradation [8], chelating agents [9], and/or oxidizing agents [1].

The use of lytic polysaccharide monooxygenases (LPMOs) has been an emerging focal point for research on the enzymatic degradation of polysaccharides. These enzymes are classified as auxiliary activity (AA) enzymes in the Carbohydrate-Active enZyme database (CAZy; http://www.cazy.org) [10], corresponding to families AA9 (originally GH61), AA10 (originally CBM33), AA11, and AA13 [11]. LPMOs are copper-dependent enzymes that are capable of catalyzing the cleavage of glycosidic bonds through oxidation of sugar residues. While oxygen-dependent enzymes were already suggested as an important part of fungal cellulose degradation as early as the 1970s [12], investigation into their importance and functional impact on cellulose degradation has risen dramatically in recent years. Over the past decade, there has been a wide array of research into these enzymes, with current research demonstrating LPMO activity on a variety of substrates including cellulose [13], xylan [14], xyloglucan [15], chitin [16], starch [17], β-glucan [18],

Abbreviations: LPMO, lytic polysaccharide monooxygenase; MALDI, matrix assisted laser desorption ionization; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detection; PASC, phosphoric acid swollen cellulose; DP, degree of polymerization.

* Corresponding author at: DuPont™ Nutrition Biosciences ApS, Edwin Rahrs Vej 38, Brabrand, 8220, Denmark.
E-mail addresses: brian.pierce@dupont.com, bpierce72@gmail.com (B.C. Pierce), jaag@kt.dtu.dk (J.W. Agger), jesper.wichmann@dupont.com (J. Wichmann), am@kt.dtu.dk (A.S. Meyer).

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glucomannan [15], and lichenan [15]. To date, LPMOs have been shown to oxidize at the C1 position [19], the C4 position [20], or at both the C1 and C4 positions [21] of polysaccharide sugar residues to result in glycosidic cleavage and degradation of the polysaccharide [22].

One of the main drivers of the growth in research for LPMOs has been their demonstrated ability to boost the activity of traditional glycoside hydrolases (GHs) in the degradation of recalcitrant polysaccharides [11]. This has been particularly relevant for biomass conversion, where there is a strong desire to maximize the overall efficiency of the enzymes used in order to minimize production costs [23]. This enhancing effect has been demonstrated for a wide variety of substrates including cellulose [24], lignocellulose [23], spruce [25], kenaf and oak [26]. The current understanding is that this GH boosting effect is the result of the LPMOs introduction of chain breaks in the crystalline and less accessible portions of the substrate [25].

The filamentous fungi *Trichoderma reesei* (*Hypocrea jecorina*) is one of the most well studied cellulolytic systems in nature, and it is known to produce two family AA9 LPMOs, *Tr*Cel61A and *Tr*Cel61B. *Tr*Cel61A was first investigated through expression in *S. cerevisiae* in the late 1990s and was characterized as a weakly acting endoglucanase at that time [27]. Due to this observed activity, it was given the name EGIV and this activity was also confirmed through homologous expression in *T. reesei* [28]. The LPMO activity of *Tr*Cel61A was confirmed through recombinant expression in *Pichia pastoris* by Tanghe, et al. [29]. In the latter study, both neutral and oxidized cello-oligosaccharides were observed following *Tr*Cel61A treatment of phosphoric acid swollen cellulose (PASC). Of the oxidized products, C1 oxidation was the most abundant; however evidence of C4 and C1-C4 oxidation led to their classification of *Tr*Cel61A as a type-3 LPMO [21].

In this study, the LPMO activity of *Tr*Cel61A expressed in *T. reesei* was investigated against a range of substrates and the oxidation products of these treatments were analyzed with MALDI-TOF, ESI mass spectrometry and high performance anion exchange chromatography (HPAEC). The effect of *Tr*Cel61A addition on endoglucanase treatment of soy spent flake was also investigated, and a synergistic effect was observed resulting in increased glucose yields compared to enzyme addition alone. We present, to our knowledge for the first time, demonstration of LPMO activity on soybean cellulosic polysaccharides and the utility of this activity in the improved enzymatic degradation of this abundant and underutilized raw material.

2. Materials and methods

2.1. Materials

Soy spent flakes were obtained from DuPont™ Nutrition & Health – Protein Solutions (St. Louis, MO) as a by-product of the soy protein isolate production process. The material was lyophilized and ground to pass a USA-Standard ASTM 40-mesh screen. Avicel® PH-101 microcrystalline cellulose, L-fucose (Fuc), L-rhamnose (Rha), D-arabinose (Ara), D-galactose (Gal), D-glucose (Glc), D-xylose (Xyl), D-glucuronic acid (GlcA), D-galacturonic acid (GalA), D-(+)-cellobiose, D-(+)-cellotriose, cellotetraose, cellopentaose, and cellohexaose were obtained from Sigma-Aldrich (St. Louis, MO). Galactan (potato) was obtained from Megazyme International (County Wicklow, Ireland). GE Healthcare PD MidiTrap G-25 columns were purchased from VWR (Radnor, PA). Maltodextrin 20DE (item no. 019046) was purchased from Cargill (Wayzata, MN). PASC was obtained from DuPont™ Industrial Biosciences (Palo Alto, CA) and was prepared as described by Wood [30].

2.2. Chemicals

Concentrated sodium hydroxide (50%; NaOH), 96% glacial acetic acid, sodium acetate trihydrate, Tris base, copper(II) sulfate pentahydrate, 2,5-dihydroxybenzoic acid MALDI-MS matrix, methanol, acetonitrile, urea, ammonium bicarbonate, DL-dithiothreitol (DTT), iodoacetamide, formic acid, and trifluoroacetic acid (TFA) were all purchased from Sigma-Aldrich (St. Louis, MO). Concentrated sulfuric acid (95–98% w/v; H_2SO_4) was purchased from VWR (Radnor, PA) and used to generate a 12 M H_2SO_4 stock solution. Concentrated hydrochloric acid (30%; HCl) was purchased from Merck Millipore (Darmstadt, Germany) and used to generate a 2 M HCl solution. L(+)-Ascorbic acid AnalaR NORMAPUR® was obtained from VWR (Radnor, PA). Sodium azide (NaN_3) was purchased from Merck Performance Materials (Darmstadt, Germany).

2.3. Enzymes

Lytic polysaccharide monooxygenase *Tr*Cel61A from *Trichoderma reesei* (EGIV;UniProt:O14405; AA9) was obtained from DuPont™ Industrial Biosciences (Palo Alto, CA) following recombinant expression in a *Trichoderma reesei* (*Hypocrea jecorina*) strain deleted for the major cellulase genes and beta-glucosidase as described in PCT Pat. Appl. Publ. No. WO2009/048488 [31]. Cell debris was removed by centrifugation and the supernatant was concentrated using a VivaFlow50 ultrafiltration membrane (MWCO 30 kDa) from Sartorius (Göttingen, Germany), resulting in a final protein concentration of 100 mg/mL. The purity of the enzyme was assessed using an SDS-PAGE gel (Supplementary Information Fig. S1), and N-terminal characterization was performed as described in 2.5.5 to verify the expected presence of an N-terminal histidine with methylation of the imidazole [18]. Endo-1,4-β-D-glucanase from *Bacillus amyloliquefaciens* (EC 3.2.1.4; GH5) with 3500U activity (17.07 mg/mL protein) and beta-glucosidase from *Agrobacterium sp.* (EC 3.2.1.21; GH1) with 600U activity (2.48 mg/mL protein) were obtained from Megazyme International (County Wicklow, Ireland). α-Chymotrypsin from bovine pancreas (EC 3.4.21.1) was obtained from Sigma-Aldrich (St. Louis, MO).

2.4. Soy spent flake NaOH pretreatment

NaOH pretreated soy spent flakes were produced as described in Mittal, Katahira, Himmel & Johnson [32]. In brief, dry soy spent flakes were combined with 16.5% w/w NaOH in an amount of 8.57 mg per mL of 16.5% w/w NaOH. This mixture was stirred under nitrogen at 25 °C for 2 h. Upon completion, the material was separated by centrifugation and the soluble fraction was decanted off with the remaining insoluble fraction re-diluted in MilliQ water to the original reaction volume and vortexed. The centrifugation, separation and dilution described above were repeated twelve times until the pH of the soluble fraction was neutral. The final, rinsed insoluble material was lyophilized and ground to pass a USA-Standard ASTM 40-mesh screen.

2.5. Material characterization

2.5.1. High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

The free monosaccharides in each sample were quantified by HPAEC-PAD using a Dionex ICS-3000 Ion Chromatography system with a CarboPac® PA100 guard column (50 × 2 mm) followed by a CarboPac® PA100 analytical column (250 × 2 mm) and a PAD, all from Dionex Corporation (Sunnyvale, CA). The flow rate used was 0.25 mL/min under the following elution profile, with MilliQ water as eluent A and 600 mM NaOH as eluent B: 0–18 min, 1.5% B;

18–19 min, 1.5–75% B; 19–29 min, 75% B; 29–29.1 min, 75–1.5% B; 29.1–43 min, 1.5% B. Monosaccharides were quantified using both external calibration standards containing a mixture of Fuc, Rha, Ara, Gal, Glc, Xyl, GlcA, and GalA, as well as variable internal calibration with Fuc added to each sample in the range of 6–13 ppm. The standard curves for each sugar were fitted with a linear calibration curve without offset, except for Fuc where a second order polynomial calibration curve without offset was used.

HPAEC-PAD was also used for the analysis of the oxidation products of LPMO reactions using the method of Westereng et al. [33]. This involved the use of a Dionex ICS-3000 Ion Chromatography system with a CarboPac® PA1 guard column (50 × 2 mm) followed by a CarboPac® PA1 analytical column (250 × 2 mm) and a PAD, all from Dionex Corporation (Sunnyvale, CA). The flow rate used was 0.25 mL/min under the following elution profile, with 0.1 M NaOH as eluent A and 1 M NaOAc in 0.1 M NaOH as eluent B: 0–10 min, 0–10% B; 10–35 min, 10–30% B; 35–40 min, 30–100% B (Dionex curve 6 – exponential); 40–41 min, 100–0% B; 41–50 min, 0% B.

2.5.2. Complete acid hydrolysis for monosaccharide profile

Complete acid hydrolysis to monosaccharides was conducted as described in Pierce et al. [1]. In brief, sulfuric acid (12 M) was added to the dry sample at a ratio of 6 mL/g sample and the samples were immediately vortexed to coat the dry sample in acid. The samples were incubated for 60 min in a 30 °C water bath with vortex mixing every 15 min. After incubation, the acid concentration was diluted to 0.49 M through the addition of MilliQ water. The samples were capped and autoclaved in a Tuttnauer 2840EL benchtop autoclave for 40 min at 123 °C. The samples were then diluted with MilliQ water to the required level for chromatographic analysis, filtered using 0.45 μm syringe filters, and analyzed by HPAEC-PAD as described in Section 2.5.1. All monosaccharide determination experiments were performed in duplicate.

2.5.3. Matrix-assisted laser desorption/ionization time of flight (MALDI-ToF) mass spectrometry

MALDI-ToF analysis was performed on an Ultraflex MALDI-TOF/TOF instrument (Bruker Daltonics) equipped with nitrogen 337 nm laser beam as described in Agger et al. [15]. 2 μL of sample was added to an MTP 384 ground steel target plate from Bruker Daltonics (Billerica, MA) and then 2 μL of 9 mg/mL 2,5-dihydroxybenzoic acid (DHB) in 30% acetonitrile was added to the sample, mixed, and dried under a stream of warm air. All spectra were obtained using the reflectron mode with an acceleration voltage of 25 kV, a reflector voltage of 26 and pulsed ion extraction of 40 ns in the positive ion mode. The data were collected from averaging 400 laser shots, with the lowest laser energy necessary to obtain sufficient signal to noise ratios. Instrument mass calibration was performed with Maltodextrin 20DE. Peak lists were generated from the MS spectra using Bruker flexAnalysis software (version 3.3).

2.5.4. Direct infusion electrospray ionization mass spectrometry (ESI–MS)

Mass spectrometry analysis by ESI–MS was performed on a linear ion trap LTQ Orbitrap Fusion from Thermo Scientific (Waltham, MA) coupled to a TriVersa NanoMate® chip-based electrospray device from Advion (Ithaca, NY). The analyses were done by direct infusion to the MS without chromatographic separation. Samples were prepared in a 96-well microtiter plate with a methanol:sample ratio of 1:2. The electrospray was controlled with Advion ChipSoft Manager version 8.3.1 and operated in positive mode with a gas pressure (N_2) of 0.6 psi, voltage of 1.8 kV, 5 μL tip collection, and sample temperature of 7 °C utilizing plastic tips (part no. 1004763) and a 5 μm ESI Chip® (part no. 1003446). Fluoranthene was used as an internal calibrant and the ion transfer tube temperature was set to 200 °C. The acquisition time was set to 2.0 min, with approximately 40 scans per minute for MS and MS2. MS full scans were performed in the m/z 300–1200 mass range, utilizing an Orbitrap detector at 120 K resolution and 2 microscans. MS2 scans were performed on Na-adducts with CID (collision-induced dissociation), using He as the colliding gas in the m/z 105–400 scan range with quadrupole isolation, Orbitrap detection at 15 K resolution, an isolation window of 2, 28% collision energy, and 3 microscans. MS Data from ESI–MS was processed in Xcalibur 3.0.63 from Thermo Scientific (Waltham, MA).

2.5.5. N-terminal characterization with liquid chromatography–mass spectrometry (LC–MS)

Proteomic analysis was performed using a modified spin filter chymotrypsin digestion as previously described by Manza et al. and Wiśniewski et al. [34,35]. LC separation was performed using an UltiMate 3000 Nano LC system from Dionex Corporation (Sunnyvale, CA), a 5 μm Acclaim PepMap C18 guard column (20 mm × 100 μm) from Thermo Scientific (Waltham, MA), and a 3 μm ReproSil-Pur C18-AQ column (100 mm × 75 μm) from Dr. Maisch GmbH (Ammerbuch-Entringen, Germany). The flow rate used was 300 nL/min under the following elution profile, with H_2O/formic acid (100:0.1) as eluent A and acetonitrile/formic acid (100:0.1) as eluent B: 0–3 min, 2% B; 3–21 min, 2–41% B; 21–23 min, 41–95% B; 23–29 min, 95% B; 29–31 min, 95–2% B; 31–44 min, 2% B. MS and MS2 analysis by ESI–MS was performed in positive mode on a linear ion trap LTQ Orbitrap Fusion from Thermo Scientific (Waltham, MA) with a capillary temperature of 200 °C, 2.1 kV ESI voltage, and quadrupole isolation. For MS2 analysis, both CID (collision-induced dissociation) and HCD (higher-energy collisional dissociation) fragmentation were performed. MS Data was processed in Xcalibur 3.0.63 from Thermo Scientific (Waltham, MA).

2.6. Enzymatic reactions

2.6.1. Primary enzymatic treatment

Reaction mixtures of 300 μL total volume contained 4 mg/mL substrate cellulose, 0.02% sodium azide, and 1 mM ascorbic acid in 10 mM Na-acetate, pH 6.0. *Tr*Cel61A was Cu(II) saturated by incubating 5.0 mg/mL *Tr*Cel61A in 20 mM Tris-HCl, pH 8.0 with a 3-fold molar excess of $Cu(II)SO_4$ for 30 min at room temperature with excess copper removed by PD MidiTrap G-25 filtration, as described by Loose, Forsberg, Fraaije, Eijsink, & Vaaje-Kolstad [36]. Enzymes were dosed based on protein content at levels of 0.25 and 0.05 mg/mL for *Tr*Cel61A and endoglucanase, respectively. Samples were incubated at 40 °C with 600 rpm shaking for varying times. Following the reaction, all enzyme samples were heat-inactivated through incubation at 95 °C for 5 min and centrifuged at 16,300 × g for 20 mins to isolate the supernatants from the pellets.

2.6.2. Glucose release with beta-glucosidase

Following enzymatic treatment with *Tr*Cel61A and/or endoglucanase, supernatants were treated with beta-glucosidase from *Agrobacterium* sp. to release glucose for quantification with HPAEC-PAD as described in Section 2.5.1. Beta-glucosidase was dosed based on protein at 0.05 mg/mL in 180 μL reactions containing 40% sample from the primary enzymatic treatment in 10 mM Na-acetate buffer, pH 6.0. Samples were incubated at 40 °C with 600 rpm shaking for 20 h. Following the reaction, beta-glucosidase was heat-inactivated by incubating the samples at 95 °C for 5 min and glucose release was quantified by HPAEC-PAD. Sample data was converted into substrate glucose released (%) by dividing the amount of glucose released in the enzyme treatment by the total

amount present in the original reaction substrate for the primary enzymatic treatment and multiplying by 100.

3. Results and discussion

3.1. LPMO substrate specificity

Substrate specificity was evaluated by incubating *Tr*Cel61A with a variety of substrates (PASC, Avicel®, galactan, cellopentaose, and cellohexaose) and monitoring the release of oligomers using MALDI-ToF, ESI–MS, and HPAEC-PAD. Galactan was included in this study due to its high prevalence in soybean [1] and in order to eliminate any ambiguity in the interpretation of the MS results due to the identical molar masses of glucose and galactose. In addition, cellopentaose and cellohexaose were included to determine the activity of *Tr*Cel61A on soluble cello-oligomers. *Tr*Cel61A did not demonstrate measurable evidence of activity on galactan, cellopentaose, or cellohexaose with or without the addition of ascorbic acid (results shown in Supplementary Information Figs. S2–S4).

*Tr*Cel61A did demonstrate oxidative activity on PASC (Fig. 1) in the presence of 1 mM ascorbic acid. Fig. 1A shows the MALDI-ToF identification of degree of polymerization (DP) 2–11 cello-oligomers following treatment of PASC for 24 h. For each of these DPs, a characteristic mass pattern was observed, as shown in Fig. 1B for DP4. Neutral oligomers were detected, which is unexpected for a pure LPMO that is only active on polymeric substrates; however, this is most likely the result of trace endoglucanase activity in the *Tr*Cel61A enzyme preparation. In addition to the neutral mass, three prominent masses corresponding to oxidized products were detected. For an observed neutral oligomer of mass M, *Tr*Cel61A treatment also resulted in [M−2 Da], [M+16 Da], and [M+38 Da] signals. The [M−2 Da] mass is characteristic of the ketone form of C4 oxidation at the non-reducing end of the oligomer (ex: Glc4ketoGlc$_3$ for a DP4 cello-oligomer). It should be noted that a portion of this signal could also result from the presence of the closed ring C1-oxidized lactone. The [M+16 Da] can result from two types of oxidation; the aldonic acid form of C1 oxidation at the reducing end of the oligomer (ex: Glc$_3$Glc1A for a DP4 cello-oligomer) as well as the gemdiol (hydrated ketone) form of C4 oxidation at the non-reducing end of the oligomer (ex: Glc4gemGlc$_3$ for a DP4 cello-oligomer). Finally, the [M+38 Da] signal is indicative of the double sodiated form of Glc$_3$Glc1A, where the second sodium displaces the aldonic acid proton to result in [M+2Na-H]$^+$. This mass was also observed for ESI MS, where the mass accuracy confirms that this is in fact [M+2Na-H]$^+$ and not the K-adduct of the native mass, M. The high abundance of both [M−2 Da] and [M+16 Da] in the MALDI spectra strongly indicates the presence of both C1 and C4 oxidation. In the low-water MALDI environment, the majority of C4 oxidized compounds will exist in their [M−2 Da] ketone oxidized form, leaving the [M+16 Da] peak to consist primarily of the C1 oxidized aldonic acid. In addition, a discrete [M+14 Da] peak corresponding to the C1-C4 doubly oxidized products of the reaction (ex: Glc4ketoGlc$_2$Glc1A for a DP4 cello-oligomer), was observed in the MALDI spectra for DP4-6 oligomers.

Fig. 1C shows the ESI MS-MS CID fragmentation of the sodiated and oxidized DP2 [M+16-Na]$^+$ ion (m/z 381.1004) using the nomenclature described by Domon and Costello [37]. Fig. 2 depicts the sodium adducts from MS-MS fragmentation for both the C1 oxidized disaccharide GlcGlc1A and the C4 oxidized disaccharide Glc4gemGlc, as described by Isaksen et al. [20]. The spectrum in Fig. 1C shows several ions that are highly characteristic of C4 oxidation: the disaccharide readily loses the mass of two water molecules (m/z 363.09 and 345.08); fragmentation m/z ions of $^{0,2}A_2$-H_2O (m/z 303.07) and $^{3,5}A_2$ (m/z 275.07); a prominent Y_1 peak (m/z 203.05); as well as characteristic loss of water in the B_1 fragment (m/z 183.02) (Fig. 2). The ESI MS-MS also shows fragments indicative of C1 oxidation, such as the characteristic loss of a carboxyl group ($^{(0),1}A_2$; m/z 335.09) and the presence of the Y_1 fragment (m/z 219.05). This latter fragment is expected to correspond primarily to C1 oxidation due to the ready dehydration of the C_1 fragment of C4 oxidation, which has been marked in parenthesis in Fig. 1C to indicate this effect. These results suggest that the samples contain a mixture of C1 and C4 oxidized products, also illustrated by the MALDI-ToF data. Note that although they appear equally abundant due to image zooming, the peak at m/z 364.09 is simply the ^{13}C-isotope peak of the m/z 363.09 signal. Fig. 1D shows the HPAEC-PAD profile of the *Tr*Cel61A treated PASC with labeling of the DP1-6 cello-oligomers. Signals are observed in the characteristic neutral, C1 oxidized, C4 oxidized, and C1-C4 oxidized regions of this chromatogram as described by Westereng et al. [38]. These results correlate well to the MS data in Fig. 1B and C, but do show differences from those reported by Tanghe et al. in their study of PASC treated with *Tr*Cel61A expressed in *Pichia pastoris* [29]. While they did observe neutral, C1 oxidized, C4 oxidized, and C1-C4 oxidized compounds in their chromatograms, they saw much fewer signals corresponding to C4 oxidation. Our data suggests that the C4 oxidized products following PASC treatment with *Tr*Cel61A are at least as abundant as the C1 oxidized products. As described in Section 2.3, we have confirmed the presence of methylation on the N-terminal histidine for the *Tr*Cel61A utilized in this study. While the effects of this methylation are still not fully understood [39], it has been proposed that the presence of this alkyl substituent may impact the electrostatic interaction between the enzyme and its substrate [40]. While Tanghe et al. do not unequivocally report on the absence of N-terminal histidine methylation in their work [29], it is known that *Pichia pastoris* lacks the cellular machinery to perform this modification [11]. Therefore, one possible explanation for the observed differences in oxidation between these two studies could be differences in the presence of this methylation. Nevertheless, our results do confirm their conclusion that *Tr*Cel61A can be classified as a type-3 LPMO [21].

Note that the presence of short cello-oligosaccharides in samples treated with *Tr*Cel61A alone (Fig. 1A) indicates either the presence of trace glucanase activity in the *Tr*Cel61A sample, or the ability of *Tr*Cel61A to act on short oligomers. However, the lack of observed activity through direct *Tr*Cel61A treatment of soluble cello-oligomers of DP5 and DP6 suggests that this is in fact a result of trace glucanase activity in the *Tr*Cel61A. The enzyme's lack of activity on short oligomers is also supported by the fact that *Tr*Cel61A appears to favor the formation of larger (DP4+) oxidation products, with these compounds in higher abundance than the DP2 and DP3 oxidation products (Fig. 1A). In addition, the presence of cello-oligosaccharides in samples treated with *Tr*Cel61A in the absence of ascorbic acid also indicates trace glucanase activity that is not a result of *Tr*Cel61A itself. Lastly, all of the oxidation effects described above for PASC were absent in control samples treated with *Tr*Cel61A in the absence of ascorbic acid (results shown in Supplementary Information Figs. S5 and S6).

In comparison to the PASC treatment, *Tr*Cel61A treatment of Avicel® showed low levels of detectable oxidative activity. Low-intensity peaks were observed in the HPAEC-PAD characteristic regions of C1 and C4 oxidation (Supplementary Information Fig. S7) [38]. In addition, low levels of the oxidized products [M−2 Da], [M+16 Da], and [M+32 Da] were detected with ESI–MS for the DP2 and DP3 cello-oligomers (Supplementary Information Fig. S8). However, these oxidized products were in very low abundance and the presence of this oxidation could not be verified with MALDI-ToF (Supplementary Information Fig. S9). For this reason, we conclude that *Tr*Cel61A treatment of Avicel® shows low detectable activity and Avicel® is therefore not a preferred substrate for this enzyme.

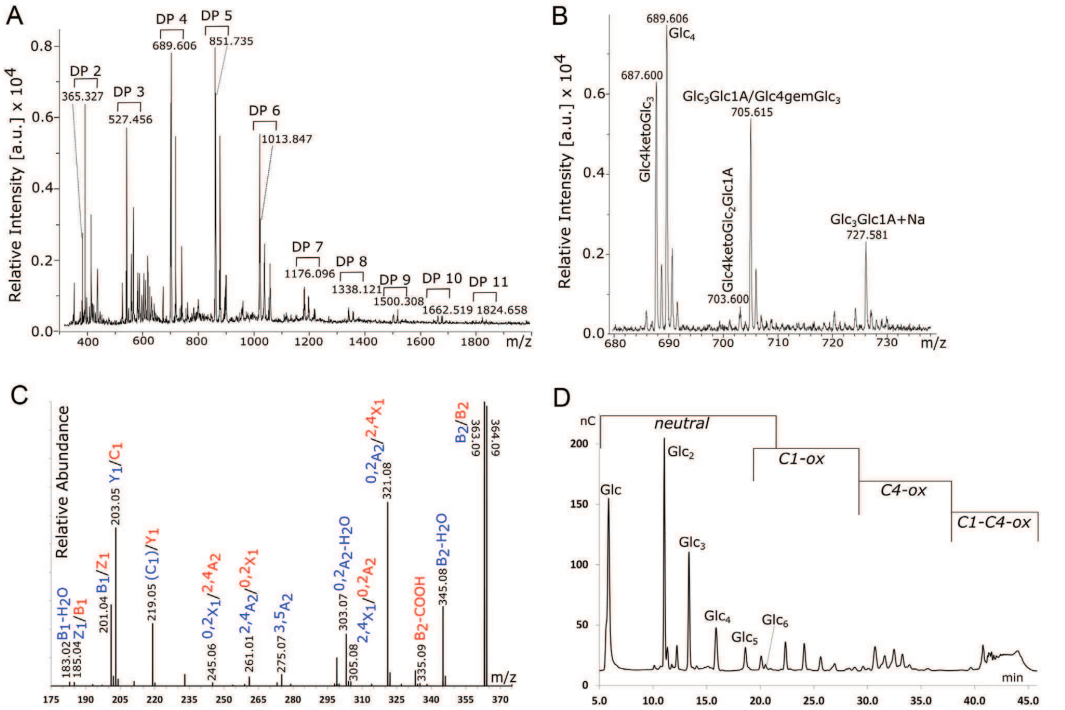

Fig. 1. *Tr*Cel61A degradation of PASC after 24 h in the presence of 1 mM ascorbic acid. All MS *m*/*z* in A-C are shown as sodium adducts. A) MALDI-ToF spectra showing the presence of DP2-11 oligomers B) MALDI-ToF spectra close up of the DP4 region demonstrating evidence of both C1, C4, and C1-C4 oxidation with relevant masses labeled: C4-ketone oxidation ($Glc4ketoGlc_3$) [M−2 Da], non-oxidized oligomer (Glc_4) [M Da], C1-C4 ketone and aldonic acid oxidation ($Glc4ketoGlc_2Glc1A$) [M+14 Da], C1-aldonic acid oxidation (Glc_3Glc1A) [M+16 Da], C4-gemdiol oxidation ($Glc4gemGlc_3$) [M+16 Da], and double sodiated C1-aldonic acid oxidation (Glc_3Glc1A + Na) [M+38 Da] C) ESI MS-MS of the *m*/*z* 381.1004 peak, corresponding to the DP2 [M+16 Da] mass. Fragments assigned and labeled as per Fig. 2 with labeling corresponding to Glc4gemGlc, C4 oxidized (blue) and GlcGlc1A, C1 oxidized (red) products. Parenthesis indicate an unlikely product D) HPAEC-PAD profile showing the presence of neutral and C1, C4, and C1-C4 oxidized degradation products as per Westereng et al. [38]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

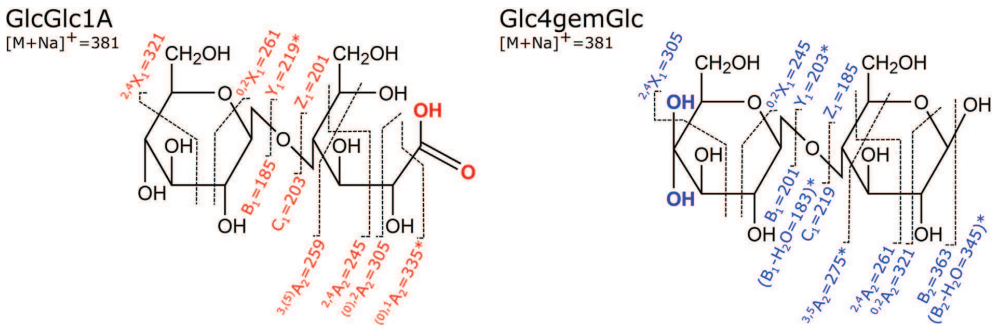

Fig. 2. MS-MS fragmentation products of the C1 oxidized disaccharide (aldonic acid) GlcGlc1A (red) and the C4 oxidized disaccharide (gemdiol) Glc4gemGlc (blue). Fragments characteristic of either C1 or C4 oxidation are highlighted by an asterisk. All masses given are for sodium adducts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. LPMO activity on soy spent flake

The effect of *Tr*Cel61A treatment on soy spent flake was investigated through *Tr*Cel61A incubation and monitoring of the release of cello-oligomers using MALDI-ToF, ESI–MS, and HPAEC-PAD. As described in 3.1, *Tr*Cel61A showed low detectable activity on the crystalline cellulose substrate Avicel®. For this reason, two forms of soy spent flake were investigated in this study: the native spent flake and spent flake treated with 16.5% (w/w) NaOH. This NaOH pretreatment is known to convert the cellulose I allomorph into cellulose II, as well as form a less crystalline cellulose [32], an effect that has been shown to affect the rate of cellulose enzymatic hydrolysis [41]. The NaOH pretreated spent flakes were found to contain significantly more glucose (constituting more than 50 molar% of

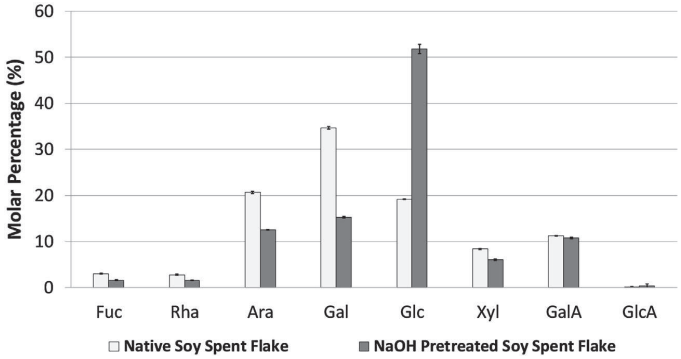

Fig. 3. Monomeric composition of native soy spent flake and NaOH pretreated soy spent flake as determined by complete acid hydrolysis with HPAEC-PAD measurement. Data are shown as averages of duplicate analyses of each sample with standard deviations given by error bars.

the total carbohydrates), and less arabinose and galactose moieties than the native soy spent flakes (Fig. 3). This change in monosaccharide composition after NaOH pretreatment results in a reduction of substrate hemicellulose, and is expected to lead to increased accessibility of the cellulose during enzymatic treatment.

*Tr*Cel61A showed no ability to oxidize the native soy spent flake. However, *Tr*Cel61A incubation with NaOH treated spent flake resulted in oxidative degradation in the presence of 1 mM ascorbic acid (Fig. 4). The analysis shows similar results to those seen from *Tr*Cel61A treatment of PASC (Fig. 1). Fig. 4A shows the MALDI-ToF identification of DP2-7 cello-oligomers, a smaller range than that observed for PASC. Each of these DPs shows the same characteristic mass pattern that was observed with PASC, as shown in Fig. 4B for DP4. Again, for an observed neutral oligomer of mass M, *Tr*Cel61A treatment resulted in [M−2 Da], [M+16 Da], and [M+38 Da] signals corresponding, most prominently, to the ketone C4 oxidiation, aldonic acid C1 oxidiation, and double sodiated aldonic acid C1 oxidiation, respectively. Note that the [M+14 Da] signals corresponding to C1-C4 doubly oxidized oligomers were not observed in the MALDI-ToF spectrum.

Fig. 4C shows the ESI MS-MS CID fragmentation of the sodiated DP2 $[M+16\text{-}Na]^+$ ion (m/z 381.1004) using the nomenclature of Domon and Costello [37]. Note that with this mass accuracy it is possible to confirm that this peak does not represent the K-adduct of the native oligomer, M. Just as with *Tr*Cel61A treatment of PASC, this spectrum shows characteristic ions of C4 oxidation: $B_2\text{-}H_2O$, 345.08; $^{0,2}A_2\text{-}H_2O$, 303.07; $^{3,5}A_2$, 275.07; a prominent Y_1 peak, 203.05; and $B_1\text{-}H_2O$, 183.00 (Fig. 2). Unlike in PASC, the NaOH pretreated spent flakes do not show the presence of the $^{0,2}X_1$ (m/z 245.06) ion or the $^{(0),1}A_2$ (m/z 335.09) ion indicative of C1 oxidation; however, the C1 oxidation fragmentation products $^{3,5}X_1$ (m/z 307.14) and Y_1 (m/z 219.05) are observed. Again, it is expected that this latter fragment corresponds primarily to C1 oxidation due to the dehydration of the C_1 fragment of C4 oxidation. Fig. 4D shows the HPAEC-PAD profile of the *Tr*Cel61A treated NaOH pretreated spent flakes. Signals are observed in the neutral, C1 oxidized, C4 oxidized, and C1-C4 oxidized regions of the chromatogram; however, the overall signal intensity is lower than that observed from PASC treatment. The signals observed in the double oxidized region are also observed for controls run in the absence of ascorbic acid (results given in Supplementary Information Fig. S10), indicating that only C1 and C4 oxidation alone are detectable for this treatment. This is confirmed a lack of signal for C1-C4 oxidation in the MALDI-ToF spectrum. One possible explanation for this difference is the lower signal to noise ratio obtained in the MALDI-ToF of the *Tr*Cel61A treated NaOH pretreated spent flakes (Fig. 4D), which could obscure the presence of the less-abundant C1-C4 doubly oxidized products. However, these signals were also not observed using higher resolution ESI–MS (data not shown) or HPAEC-PAD, suggesting that these products are indeed not present.

As previously mentioned, the presence of short cello-oligosaccharides in Fig. 4A suggests trace glucanase activity in the *Tr*Cel61A preparation. All oxidation effects described above were absent in control samples treated with *Tr*Cel61A in the absence of ascorbic acid (results shown in Supplementary Information Figs. S10 and S11). In addition, it is important to note that although the NaOH pretreated spent flakes contain a significant amount of galactose resulting from the type I arabinogalactan in the native spent flakes [1], the lack of observed *Tr*Cel61A activity on galactan suggests that the enzyme has no activity on this class of polysaccharide. However, it should be noted that the lack of activity on pure galactan does not strictly preclude the possibility of galactan activity in the presence of more complex polysaccharides, as Frommhagen et al. have demonstrated a dependence between the presence of cellulose and the ability of *Mt*LPMO9A from *Myceliophthora thermophila* C1 to oxidize xylan [14]. For this reason, we cannot completely rule out the possibility of galactan oxidation, however the lack of observed *Tr*Cel61A activity on native soy spent flakes does indicate that *Tr*Cel61A does not form oxidized galactooligosaccharides.

3.3. Synergistic effect between LPMO and endoglucanase on soy spent flakes

The effect of *Tr*Cel61A treatment of NaOH pretreated soy spent flake on cellulose hydrolysis was investigated through a time course study spanning incubation times of 0–72 h. Samples were incubated with varying combinations of *Tr*Cel61A and/or *endo*-1,4-β-D-glucanase from *Bacillus amyloliquefaciens* (EG). Following treatment and heat-inactivation of the enzymes, the soluble fractions were treated with beta-glucosidase from *Agrobacterium* sp. (BG) in order to quantify the enzymatically solubilized cello-oligomers as glucose via HPAEC-PAD analysis. As demonstrated in 3.2, the presence of C1 and C4 oxidized glucose monomers are expected in products treated with *Tr*Cel61A. For a sample containing both *Tr*Cel61A and EG, the oxidized oligomers present would result in DP2 oxidized fragments, with the post reaction BG treatment unable to degrade the modified non-reducing end of any C4 oxidized compounds. However, the abundance of DP2 fragments with C4 oxidation is expected to be negligible in comparison to the non-oxidized glucose monomers generated in the reactions from EG and/or BG treatment.

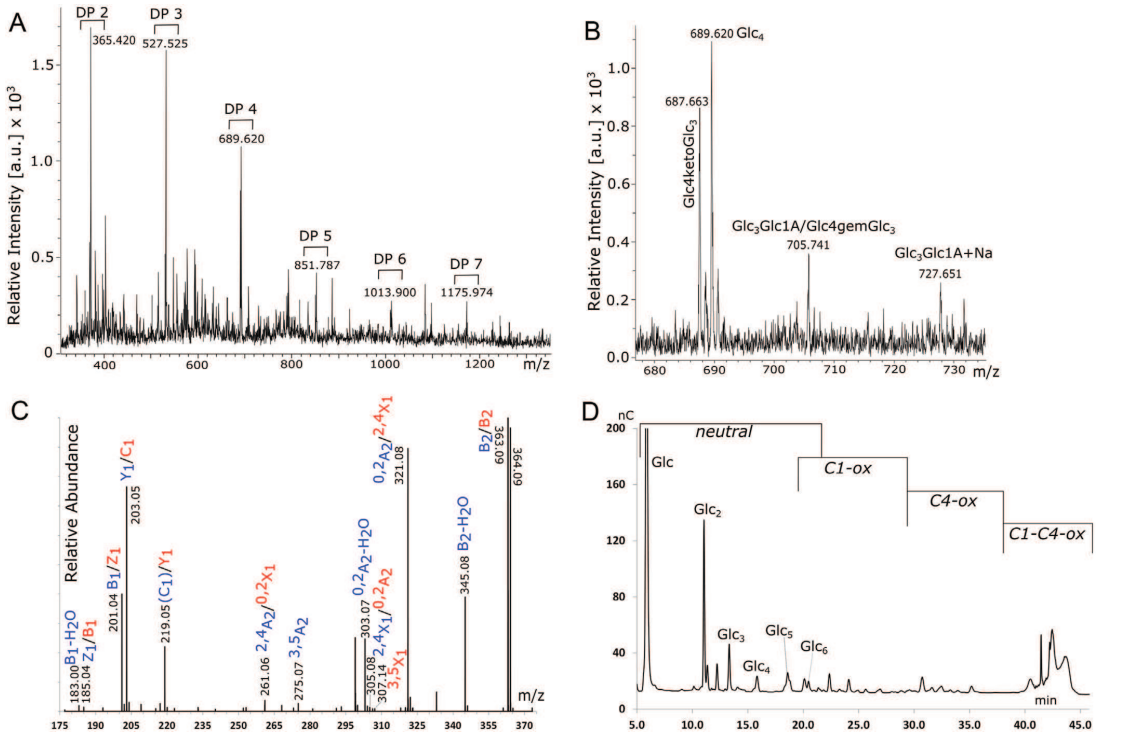

Fig. 4. *Tr*Cel61A degradation of NaOH pretreated soy spent flakes after 24 h in the presence of 1 mM ascorbic acid. All MS *m/z* in A-C are shown as sodium adducts. A) MALDI-ToF spectra showing the presence of DP2-7 oligomers B) MALDI-ToF spectra close up of the DP4 region showing evidence of both C1 and C4 oxidation with relevant masses labeled: C4-ketone oxidation ($Glc4ketoGlc_3$) [M−2 Da], non-oxidized oligomer (Glc_4) [M Da], C1-aldonic acid oxidation (Glc_3Glc1A) [M+16 Da], C4-gemdiol oxidation ($Glc4gemGlc_3$) [M+16 Da], and double sodiated C1-aldonic acid oxidation ($Glc_3Glc1A+Na$) [M+38 Da] C) ESI MS-MS of the *m/z* 381.1004 peak, corresponding to the DP2 [M+16 Da] mass. Fragments assigned and labeled as per Fig. 2 with labeling corresponding to Glc4gemGlc, C4 oxidized (blue) and GlcGlc1A, C1 oxidized (red) products. Parenthesis indicate an unlikely product D) HPAEC-PAD profile showing the presence of neutral and C1 and C4 oxidized degradation products as per Westereng et al. [38]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Glucose released (%) from NaOH pretreated soy spent flakes following treatment with *Tr*Cel61A and/or *endo*-1,4-β-D-glucanase from *Bacillus amyloliquefaciens* (EG). Standard deviations of duplicate analyses given in parentheses. Values are significantly different ($p < 0.05$) from values in the same column if followed by different letters (a-c). The theoretical sum value represents the glucose release expected from the *Tr*Cel61A and EG treatments alone, and has been included for ease of reference.

Sample Treatment	Substrate Glucose Released (%)			
	8 h	24 h	48 h	72 h
*Tr*Cel61A	1.90[a] (±0.05)	4.17[a] (±0.59)	5.28[a] (±2.83)	7.45[a] (±0.22)
EG	13.99[b] (±4.07)	16.71[b] (±4.17)	17.80[b] (±3.88)	19.77[b] (±3.62)
*Tr*Cel61A + EG	17.00[b] (±2.29)	25.13[c] (±3.23)	29.37[c] (±2.05)	34.48[c] (±4.84)
Theoretical Sum	*15.89*	*20.88*	*23.08*	*27.22*

Table 1 displays the results of these trials in percentage of the substrate glucose detected via HPAEC. *Tr*Cel61A alone showed low levels of glucose release (1.90–7.45%) from 8 to 72 h, as expected due to the contaminating endoglucanase activity observed in 3.1. Incubation of NaOH pretreated soy spent flakes with a 5-fold increase in *Tr*Cel61A did not result in any significant increase in glucose release when compared to the standard *Tr*Cel61A treatment (results not shown), indicating that *Tr*Cel61A is not limiting during the reaction. EG treatment alone was capable of releasing just over 19.5% of the substrate glucose content after 72 h, whereas the combination of *Tr*Cel61A and EG resulted in significant increases in the levels of glucose released. After 72 h, the combination of *Tr*Cel61A and EG was able to liberate 34% of the substrate glucose, a 74% increase when compared with EG treatment alone. It is also worthwhile to compare this result with the observed glucose release from each enzyme individually, given as the theoretical sum in Table 1, in order to quantify the synergistic effect when these enzymes are added in combination. When this is done, we observe that the combination of *Tr*Cel61A and EG represents a 27% increase in glucose release after 72 h compared to the expected glucose levels from treatment with each enzyme alone. Removing the reductant from the reaction resulted in the loss of this synergistic effect, demonstrating that the oxidative capabilities of *Tr*Cel61A are responsible for this boost in efficiency (results shown in Supplementary Information Table S1).

It is instructive to compare the relative rates of glucose release between the samples in Table 1. For the *Tr*Cel61A and EG treatments alone, the rates of glucose release over the reaction period (8–72 h)

are 0.081 and 0.084%/hr, respectively. In comparison, the rate of glucose release for the *Tr*Cel61A + EG combination is 0.257%/hr, which is approximately 3.2 times larger. This difference in rates also demonstrates the synergistic effect created by the combination of these two enzymes. In this study, the effects of BG addition directly to the primary enzyme reactions was also investigated. While we hypothesized that BG hydrolysis of the major EG reaction product, cellobiose, could lead to increased EG productivity in the reaction [42], no significant evidence of increased glucose release by concurrent BG treatment could be observed in these studies (data not shown). It is also worth noting that the continued activity observed for *Tr*Cel61A over the 72 h period in these reactions is unusual for an LPMO, which often stop functioning earlier in the reaction due to H_2O_2 inactivation. One explanation for this observation is that the side reaction generating H_2O_2 is limited in the presence of excess substrate as described by Kittl et al. [43].

4. Conclusions

In this article, we have shown that *Tr*Cel61A results in oxidative degradation of both PASC and NaOH pretreated soy spent flakes. We have demonstrated low detectable activity on Avicel® crystalline cellulose, but no activity on native soy spent flake, galactan, or DP5-6 soluble cello-oligomers. Through analysis with MALDI-ToF, ESI–MS, and HPAEC we have provided clear evidence that *Tr*Cel61A is capable of oxidizing at the C1 and C4 positions in both PASC and NaOH pretreated soy spent flakes. In addition, the boosting effect of *Tr*Cel61A on endoglucanase hydrolysis was described. The addition of *Tr*Cel61A to endoglucanase treatment resulted in a 27% increase in substrate glucose release for NaOH pretreated soy spent flakes after 72 h when compared to the release by the individual enzymes. While additional studies are required to determine the suitability of other LPMOs to degrade this complex substrate, the current results suggest promising new opportunities for the future use of LPMOs in the enzymatic degradation of soy polysaccharides.

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The author contributions are as follows: B. C. P. contributed to the study design, analysis and interpretation of the results as well as the writing of the manuscript. J. W. A. contributed to the MS analysis, study design, overall data interpretation, and manuscript revision. J. W. and A. S. M. contributed to the study design, data interpretation, manuscript revision, and project supervision.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enzmictec.2016.12.007.

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A comparative study on the activity of fungal lytic polysaccharide monooxygenases for the depolymerization of cellulose in soybean spent flakes

Brian C. Pierce [a, b, c, *], Jane Wittrup Agger [b], Zhenghong Zhang [d, e], Jesper Wichmann [a], Anne S. Meyer [b]

[a] DuPont™ Nutrition Biosciences ApS, Edwin Rahrs Vej 38 Brabrand, 8220, Denmark
[b] Department of Chemical and Biochemical Engineering, Center for Bioprocess Engineering, Technical University of Denmark, Søltofts Plads, Building 229, Kgs. Lyngby, 2800, Denmark
[c] DuPont™ Nutrition & Health - Protein Solutions, 4300 Duncan Ave., Saint Louis, MO, 63110, USA
[d] DuPont™ Industrial Biosciences, China Research Center, Building 10, Lane 280, Linhong Road, Shanghai, 200335, PR China
[e] State Key Laboratory of Microbial Metabolism, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai, 200240, PR China

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ABSTRACT

Lytic polysaccharide monooxygenases (LPMOs) are copper-dependent enzymes capable of the oxidative breakdown of polysaccharides. They are of industrial interest due to their ability to enhance the enzymatic depolymerization of recalcitrant substrates by glycoside hydrolases. In this paper, twenty-four lytic polysaccharide monooxygenases (LPMOs) expressed in *Trichoderma reesei* were evaluated for their ability to oxidize the complex polysaccharides in soybean spent flakes, an abundant and industrially relevant substrate. *Tr*Cel61A, a soy-polysaccharide-active AA9 LPMO from *T. reesei*, was used as a benchmark in this evaluation. In total, seven LPMOs demonstrated activity on pretreated soy spent flakes, with the products from enzymatic treatments evaluated using mass spectrometry and high performance anion exchange chromatography. The hydrolytic boosting effect of the top-performing enzymes was evaluated in combination with endoglucanase and beta-glucosidase. Two enzymes (*Tr*Cel61A and Aspte6) showed the ability to release more than 36% of the pretreated soy spent flake glucose – a greater than 75% increase over the same treatment without LPMO addition.

1. Introduction

Soybean polysaccharides are produced in large quantities globally as an industrial by-product of soy protein, soy milk, and tofu production. When resulting from tofu or soy milk production they are generally referred to as okara, and when generated as a by-product of soy protein isolate production they are known as spent flakes [1]. These materials are predominantly composed of high molar mass, water-insoluble cell wall polysaccharides including type I arabinogalactan, cellulose, arabinoxylan, rhamnogalacturonan, arabinan, xyloglucan, and homogalacturonan [2]. Each year, approximately 3.91×10^6 tons of soybean polysaccharides are produced through Chinese, Japanese and Korean tofu production, with the vast majority of this material disposed of as waste or burned [3–5]. This disposal not only adds significant costs to the production process, but also raises environmental sustainability concerns. It is therefore of significant interest to develop alternate

Abbreviations: LPMO, lytic polysaccharide monooxygenase; HPAEC, high performance anion exchange chromatography; PASC, phosphoric acid swollen cellulose; DP, degree of polymerization.

* Corresponding author. DuPont™ Nutrition & Health - Protein Solutions, 4300 Duncan Ave., Saint Louis, MO, 63110, USA.
E-mail addresses: brian.pierce@dupont.com (B.C. Pierce), jaag@kt.dtu.dk (J.W. Agger), zhenghong.zhang@dupont.com (Z. Zhang), jesper.wichmann@dupont.com (J. Wichmann), am@kt.dtu.dk (A.S. Meyer).

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applications for improved utilization of this abundant industrial by-product.

Lytic polysaccharide monooxygenases (LPMOs) are copper-dependent enzymes capable of polysaccharide depolymerization through oxidation of sugar residues [6]. They have been classified as auxiliary activity (AA) enzymes in the Carbohydrate-Active enZyme database (CAZy; http://www.cazy.org) [7], and are currently categorized into four families: AA9 (formerly GH61), AA10 (formerly CBM33), AA11, and AA13. AA9 comprises fungal LPMOs active on lignocellulose, AA10 predominantly comprises LPMOs of bacterial and viral origin cleaving cellulose and chitin, AA11 comprises fungal LPMOs that cleave chitin, and AA13 comprises fungal LPMOs that cleave starch [8]. The importance of this oxidative mechanism in cellulose degradation was first suggested in 1974 by Eriksson et al. [9]. In 2010, Vaaje-Kolstad et al. described the oxidative mode of action of LPMOs and demonstrated their capacity to boost the enzymatic degradation of chitin [10]. There has been a significant amount of research into these enzymes, with LPMO activity being demonstrated for a variety of substrates including cellulose, xylan, xyloglucan, glucomannan, lichenan, starch, β-glucan, and soluble cello-oligosaccharides [11–16]. The precise oxidative mode of action of LPMOs is still under debate, but has been postulated to involve internal electron channels [17] or, more recently, Fenton-type H_2O_2 chemistry [18]. The majority of LPMO oxidations have been observed at the C1 or C4 positions of glucose [6,19], however oxidation at the C6 position has also been suggested [20]. A key feature of all structurally characterized LPMOs is the presence of a histidine brace at the active site. This site coordinates to the catalytic copper ion and consists of the imidazole and amino group of an N-terminal histidine, as well as the imidazole of an additional histidine [21]. LPMOs which have been expressed in fungi routinely display a characteristic Nε methylation of the N-terminal histidine imidazole nitrogen. While the role of this modification is still unclear and under debate, it has been proposed that the presence of an alkyl group on the imidazole side chain of the N-terminal histidine could enhance the electrostatic interaction between the enzyme and the substrate [22]. It has also recently been suggested that this methylation may play a role in protecting fungal LPMOs from oxidative self-destruction [18].

One reason for the recent interest in LPMOs is their ability to boost the activity of cellulolytic enzymes in the degradation of biomass [23–25]. Our previous work demonstrated the ability of the AA9 LPMO *Tr*Cel61A from *Trichoderma reesei* to oxidize the cellulosic polysaccharides of NaOH pretreated soybean spent flakes at both the C1 and C4 positions [26]. In addition, *Tr*Cel61A treatment of pretreated soybean spent flakes was shown to result in a significant increase in the glucose release observed from endoglucanase (EG) treatment of this substrate. The aim of the current study is to explore the suitability of other LPMOs to degrade this complex substrate. For this purpose, twenty-three additional fungal LPMOs from seven host organisms were expressed in a *Trichoderma reesei* (*Hypocrea jecorina*) strain deleted for select cellulase genes and beta-glucosidase [27]. Following expression, the N-terminal protein sequence was assessed in order to verify the N-terminal histidine and evaluate the presence of the N-terminal histidine Nε methylation. The activities of each LPMO were investigated against a variety of substrates (phosphoric acid swollen cellulose (PASC), native soy spent flakes, NaOH pretreated soy spent flakes, and Avicel® PH-101 microcrystalline cellulose) and oxidation products of these treatments were analyzed with ESI mass spectrometry and high performance anion exchange chromatography (HPAEC). For the LPMOs displaying activity on soy spent flakes, the effect of LPMO addition on substrate glucose release through endoglucanase (EG) and beta-glucosidase (BG) treatment was also investigated.

2. Results and discussion

2.1. Protein characterization

Table 1 provides a list of the twenty-four LPMOs utilized in this study. To date, the majority of these proteins have not yet been reported to have LPMO activity, with only four of them having an exact amino acid sequence match in the National Center for

Table 1
LPMOs utilized in this study listed with their reference ID, native organism, NCBI reference ID [29], presence of an InterPro predicted C-terminal cellulose binding domain (CBD) [30], and measured protein concentration.

LPMO ID	Native Organism	NCBI Reference ID	InterPro Predicted CBD	[Protein] (mg/mL)
*Tr*Cel61A	*Trichoderma Reesei*	UniProt:O14405	Yes	38.30
Chacr1	*Chaetosartorya cremea*	–	Yes	0.37
Chacr2	*Chaetosartorya cremea*	–	–	0.46
Aspfu1	*Aspergillus fumigatus*	–	Yes	0.38
Aspfu2	*Aspergillus fumigatus*	–	Yes	0.40
Aspfu3	*Aspergillus fumigatus*	GenBank: GAQ10694.1	–	0.65
Aspfu4	*Aspergillus fumigatus*	–	Yes	0.48
Aspfu5	*Aspergillus fumigatus*	GenBank: GAQ07946.1	–	0.55
Aspte1	*Aspergillus terreus*	–	–	0.65
Aspte2	*Aspergillus terreus*	–	Yes	0.74
Aspte3	*Aspergillus terreus*	–	–	0.56
Aspte4	*Aspergillus terreus*	–	–	0.66
Aspte5	*Aspergillus terreus*	–	Yes	0.67
Aspte6	*Aspergillus terreus*	–	Yes	0.59
Penci1	*Penicillium citrinum*	–	–	0.46
Penci2	*Penicillium citrinum*	–	–	0.25
Aspte7	*Aspergillus terreus*	GenBank: GAQ40595.1	Yes	0.35
Aspte8	*Aspergillus terreus*	–	–	0.23
Malci1	*Malbranchea cinamomea*	–	–	0.21
Malci2	*Malbranchea cinamomea*	–	–	0.36
Mycth1	*Myceliophthora thermophila*	–	–	0.16
Mycth2	*Myceliophthora thermophila*	–	Yes	0.28
Mycth3	*Myceliophthora thermophila*	–	–	0.31
Talle1	*Talaromyces leycettanus*	–	Yes	0.56

Biotechnology Information (NCBI) BLAST® non-redundant protein sequence database [28,29]. The closest matches for all twenty-four LPMOs from a BLAST® search are provided in Supplementary Information Section 2. Note that ten of the LPMOs showed high levels of sequence homology with proteins listed in the sequence database, giving 100% sequence coverage and matching scores of at least 99% identity, 99% positives, and 0 gaps. Table 1 also contains the results of an InterPro protein sequence domain analysis indicating whether a cellulose binding domain (CBD) is predicted based on the sequences of the LPMOs analyzed [30]. Supplementary Information Figure S1 shows the SDS-PAGE gels obtained for each LPMO used in this study. The presence of band spreading and increased molecular weights over those expected from the amino acid sequence demonstrate the presence of glycosylation, a common post-translational event which occurs during protein expression in fungi [31].

Protease digestion in combination with MS/MS-MS analysis was used to identify the N-terminal sequences of the twenty-four expressed LPMOs. A summary of the results from this analysis are presented in Supplementary Information Section 3. All twenty-four proteins were identified to primarily have N-terminal histidine residues. Chacr2 (G), Aspfu3 (G), Aspfu5 (G), Aspte1 (G), Aspte3 (G), Aspte4 (G), Aspte5 (A), Aspte6 (A), Penci1 (G), Penci2 (G), and Mycth3 (A) showed the presence of some proteins containing one additional N-terminal amino acid, indicated in parenthesis, resulting in the lack of an N-terminal histidine. While one LPMO (Penci2) showed a significant amount of expression with the amino acid addition, the relative abundance of these observed peptides for the other LPMOs were several orders of magnitude lower than peptides containing the N-terminal histidine. It is interesting to note that the majority of these amino acid additions occur with the presence of glycine immediately preceding the N-terminal histidine. In fact, only two protein sequences featuring a glycine prior to the expected terminal histidine lacked any observed N-terminal glycine addition (Malcil1 and *Tr*Cel61A). Aspfu1 (G), Aspfu2 (G), Aspfu3 (G), Aspfu4 (G), Aspfu5 (G), Aspte5 (G), Aspte6 (G), Aspte7 (Y), Aspte8 (Y), Mycth1 (Y), Mycth3 (T), and Talle1 (G) showed the presence of a one amino acid truncation, with the new N-terminal amino acid indicated in parenthesis. Significant levels of the truncated sequence were observed for Aspfu4, Aspte6, Aspte7, Aspte8, and Mycth1; however, in all cases the relative abundances of these observed peptides were significantly lower than the peptides containing the N-terminal histidine.

The presence of N-terminal histidine methylation was also evaluated due to its expected presence following expression in *T. reesei* [23]. The Nε methylation of the N-terminal histidine was observed for all twenty-four LPMOs in this study. It is interesting to observe that the methylation from *T. reesei* expression is observed even though leader peptides that are not endogenous to this organism were used. For the majority of proteins, the methylated N-terminal histidine was identified as the most prominent N-terminal fragment. Ten of the proteins (Aspte1, Aspte3, Aspte4, Penci2, Aspte7, Aspte8, Malci1, Malci2, Mycth2, and *Tr*Cel61A) were observed to have methylation present on all or the vast majority of their N-terminal histidine residues. However, a non-methylated N-terminal histidine was identified to varying degrees in the remaining fourteen proteins (Chacr1, Chacr2, Aspfu1, Aspfu2, Aspfu3, Aspfu4, Aspfu5, Aspte2, Aspte5, Aspte6, Penci1, Mycth1, Mycth3, and Talle1). For the vast majority of these proteins, peptides corresponding to the methylated N-terminal histidine were significantly more abundant than those of its non-methylated counterpart. However, five of the proteins (Chacr1, Aspfu1, Aspfu2, Aspfu4, and Talle1) showed a higher abundance of the non-methylated protein.

2.2. Evaluation of LPMO activity

The twenty-four LPMOs listed in Table 1 were evaluated for their ability to oxidatively cleave the polysaccharides in four substrates: PASC, native soy spent flakes, NaOH pretreated soy spent flakes, and Avicel®. The NaOH pretreatment of soy spent flakes serves to convert the cellulose I allomorph into cellulose II, in addition to reducing the cellulose crystallinity [32]. As previously reported, this NaOH pretreatment results in a reduction of substrate hemicellulose and a corresponding enrichment in cellulosic polysaccharides. The molar percentages of galactose, arabinose, glucose, and xylose for the native spent flakes are 34.65%, 20.67%, 19.16%, and 8.37%, respectively. Following NaOH pretreatment, these levels change to 15.30%, 12.56%, 51.77%, and 6.08%, respectively [26]. This reduction in substrate hemicellulose may also lead to the additional benefit of increased substrate accessibility during enzymatic treatment.

Table 2 shows the results of this substrate screening, with the lack of activity (−) or presence and degree of activity (+, ++, or +++) indicated for each enzyme. The degree of activity was assessed using ESI-MS to determine the presence of DP 2–6 oxidized oligomers following the 24 h enzyme treatment. For a given neutral oligomer of mass M, samples were evaluated for signals characteristic of LPMO oxidation such as a [M-2] signal indicating C4-ketone oxidation and a [M+16] signal characteristic of either C1-aldonic acid oxidation and/or C4-gemdiol oxidation. The results for this full in-depth analysis are provided in Supplementary Information Section 4, but have been condensed to a single activity level per enzyme for a given substrate in Table 2. Twenty-one of the LPMOs investigated in this study demonstrated some degree of oxidative activity on PASC after 24 h. The lack of observed activity on PASC for Aspte1, Mycth1, and Mycth3 may indicate that the enzyme treatment conditions utilized in this study were not sufficient for these enzymes to produce enough detectable product. While all LPMO treatments presented in this study were standardized at equivalent protein levels, the relative amount of LPMO protein in each enzyme sample may have differed due to the lack of extensive purification. For this reason, it is possible that

Table 2
LPMO activity on PASC, native soy spent flakes, NaOH pretreated soy spent flakes, and Avicel®. Legend: − = no activity observed; + = weak activity; ++ = moderate activity; +++ = strong activity.

LPMO	PASC	Native soy	NaOH soy	Avicel®
*Tr*Cel61A	+++	−	+++	−
Chacr1	+	−	−	−
Chacr2	++	−	+	++
Aspfu1	++	−	+	+
Aspfu2	++	−	−	−
Aspfu3	+++	−	−	−
Aspfu4	+++	−	+	+
Aspfu5	+++	−	+	+
Aspte1	−	−	−	−
Aspte2	+++	−	−	+
Aspte3	++	−	−	−
Aspte4	++	−	−	−
Aspte5	++	−	−	−
Aspte6	+++	−	+++	+++
Penci1	++	−	−	−
Penci2	++	−	−	−
Aspte7	+	−	−	−
Aspte8	+	−	−	−
Malci1	+	−	−	−
Malci2	++	−	−	−
Mycth1	−	−	−	−
Mycth2	+	−	−	−
Mycth3	−	−	−	−
Talle1	+++	−	++	+++

the relative levels of LPMO protein for these three enzyme treatments were too low for activity to be observed. It can be observed from Table 1 that these three LPMOs lack a predicted cellulose binding domain (CBD) at the C-terminus of the protein; however, there does not appear to be a clear correlation between the presence of a predicted CBD and activity for the LPMOs investigated in this study. It is also possible that these three proteins simply do not have LPMO activity on the substrates investigated in this study. The lack of activity cannot be directly linked to the N-terminal characterization data presented in 2.1 and Supplementary Information Section 3, as all three enzymes displayed the required N-terminal histidine following expression.

No oxidative activity was observed for any of the LPMOs on the native soy spent flakes; however, seven of the LPMOs investigated (*Tr*Cel61A, Chacr2, Aspfu1, Aspfu4, Aspfu5, Aspte6, and Talle1) did show activity on the NaOH pretreated spent flakes. This finding matches our previously reported results concerning the activity of *Tr*Cel61A on soy polysaccharides, and also indicates that the complex polysaccharides of soybean are highly resistant to oxidative degradation by LPMOs using the conditions employed in this study [26]. While the exact structural relationship between the cellulosic and hemicellulosic polysaccharides in soybean has not yet been fully characterized, it can be hypothesized from these findings that the hemicellulose is capable of shielding the cellulose from enzymatic attack by LPMOs. It is interesting to note from Table 2 that all LPMOs which showed activity on the NaOH pretreated soy spent flakes also showed strong activity on PASC and some degree of activity on the crystalline cellulose in Avicel®. This commonality between Avicel® activity and NaOH pretreated soy spent flake activity suggests a degree of similarity between these two substrates, and may be an indication of remaining cellulose crystallinity in the spent flakes despite the pretreatment employed in this study. While this trend is almost universal for the LPMOs considered in this study, it was observed that Aspte2 showed no detectable activity on NaOH pretreated spent flakes despite its observed activity on both PASC and Avicel®.

From the seven LPMOs showing activity on the pretreated spent flakes, three were selected as the top-performing enzymes (*Tr*Cel61A, Aspte6, and Talle1) for further screening based on the abundance of oxidized products detected with ESI MS. Fig. 1 shows the ESI MS-MS CID fragmentation spectra of the sodiated and oxidized $[M+16-Na]^+$ ion (m/z 381.10) following the LPMO treatment of PASC with these three LPMOs. The fragments have been labeled with the nomenclature of Domon and Costello [33], with Fig. 1D illustrating the possible fragmentation products for both the C4 oxidized gemdiol, Glc4gemGlc (blue), and the C1 oxidized aldonic acid, GlcGlc1A (red) as described by Isaksen et al. [16]. As was previously reported [26], *Tr*Cel61A demonstrates characteristics of both C1 and C4 oxidation following treatment with PASC (Fig. 1A). Characteristic C4 fragmentations include the loss of two water molecules (m/z 363.09 and 345.08); the presence of $^{0,2}A_2$-H_2O (m/z 303.07) and $^{3,5}A_2$ (m/z 275.07); the prominent Y_1 peak (m/z 203.05); and loss of water in the B_1 fragment (m/z 183.02). Characteristic C1 fragmentations include the characteristic loss of a

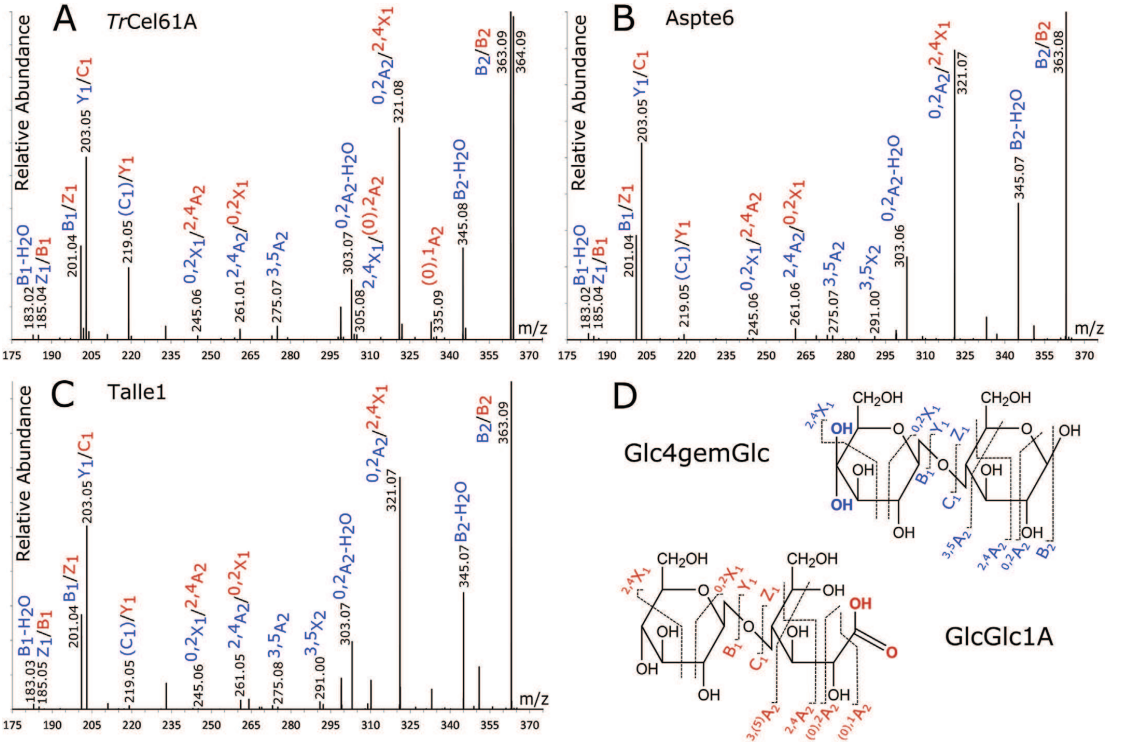

Fig. 1. ESI CID MS-MS fragmentation of the DP2 oxidized product (m/z 381.10) from LPMO treated PASC after 24 h. Masses are labeled based on expected fragmentation from the C4 oxidized product Glc4gemGlc (blue) and/or the C1 oxidized product GlcGlc1A (red). A: *Tr*Cel61A; B: Aspte6; C: Talle1; D: Expected fragmentation products from Glc4gemGlc and GlcGlc1A. Parenthesis indicate an unlikely product. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

carboxyl group ($^{(0),1}A_2$; *m/z* 335.09) and the presence of a large Y_1 fragment (*m/z* 219.05). While this latter signal could also correspond to the C_1 fragment of C4 oxidation, this fragment is expected to be in low abundance after undergoing rapid dehydration and has therefore been marked in parenthesis to indicate this effect.

Comparing Fig. 1A to the other LPMO treatments, we continue to observe the characteristic C4 oxidation fragments; however, fragmentations characteristic of C1 oxidation ($^{(0),1}A_2$; Y_1) are no longer present. This is evidenced by the lack of observed signals corresponding exclusively to C1 oxidation (red). In particular, the low signals observed for the $(C_1)/Y_1$ fragment (*m/z* 219.05) for the non-*Tr*Cel61A LPMO treatments indicate a lack of C1 oxidation. Aspte6 and Talle1 show an additional C4 oxidation fragment, $^{3,5}X_2$, not observed in the *Tr*Cel61A sample; however, they also lack the C1/C4 oxidation fragment $^{2,4}X_1/^{(0),2}A_2$, which is observed in the *Tr*Cel61A sample. In order to further explore the observation that all LPMOs showing activity on NaOH pretreated spent flakes demonstrate C4 oxidation, HPAEC-PAD profiles were run to analyze the oxidation products.

Fig. 2 shows the HPAEC-PAD chromatograms of the products produced by these three enzymes on NaOH pretreated soy spent flakes both with (black) and without (red) ascorbic acid as a reductant. In addition, the elution pattern of the neutral cello-oligomers from DP 2–6 is presented for reference (Fig. 2D). In the presence of ascorbic acid, signals are observed in the characteristic neutral (~5–22 min), C1 oxidized (~20–30 min), C4 oxidized (~30–40 min), and C1-C4 oxidized (~40–46 min) regions of this chromatogram as described by Westereng et al. [34]. The strongest signals are observed for *Tr*Cel61A and Aspte6, corresponding well to the strength of LPMO activity summarized in Table 2. Both of these enzymes show evidence of C1 and C4 oxidation (Fig. 2A and B), while Talle1 shows the majority of its signals in the C4 oxidation region (Fig. 2C). While signals are observed in the late-eluting C1-C4 oxidized region, the presence of additional signals in this region both with and without ascorbic acid makes it difficult to unambiguously confirm the presence of these doubly oxidized products.

To further investigate the observed correlation between C4 oxidation and activity on the pretreated spent flakes, additional HPAEC-PAD experiments were conducted where the soluble fractions following LPMO treatment of PASC were further treated with BG. Due to the non-reducing end activity of BG, this treatment will result in degradation of neutral and C1 oxidized soluble oligomers, but will leave the C4 oxidized compounds intact due to their modified non-reducing end. Evaluating the HPAEC-PAD chromatograms of all BG treated LPMO samples for retained signals in the C4 oxidized region (~30–40 min), we observe that seven enzymes (*Tr*Cel61A, Chacr2, Aspfu3, Aspfu4, Aspfu5, Aspte6, and Talle1) continue to show strong evidence of C4 oxidation and four enzymes (Aspfu1, Aspte2, Aspte3, and Aspte4) show weak evidence of C4 oxidation (results not shown). All other LPMOs show no signals in this region of the chromatogram, indicating that no C4 oxidation took place. Comparing these results to the activity data presented in Table 2, we observe that all LPMOs demonstrating activity on the NaOH pretreated spent flakes also show confirmed C4 oxidation. In addition, the three top-performing enzymes

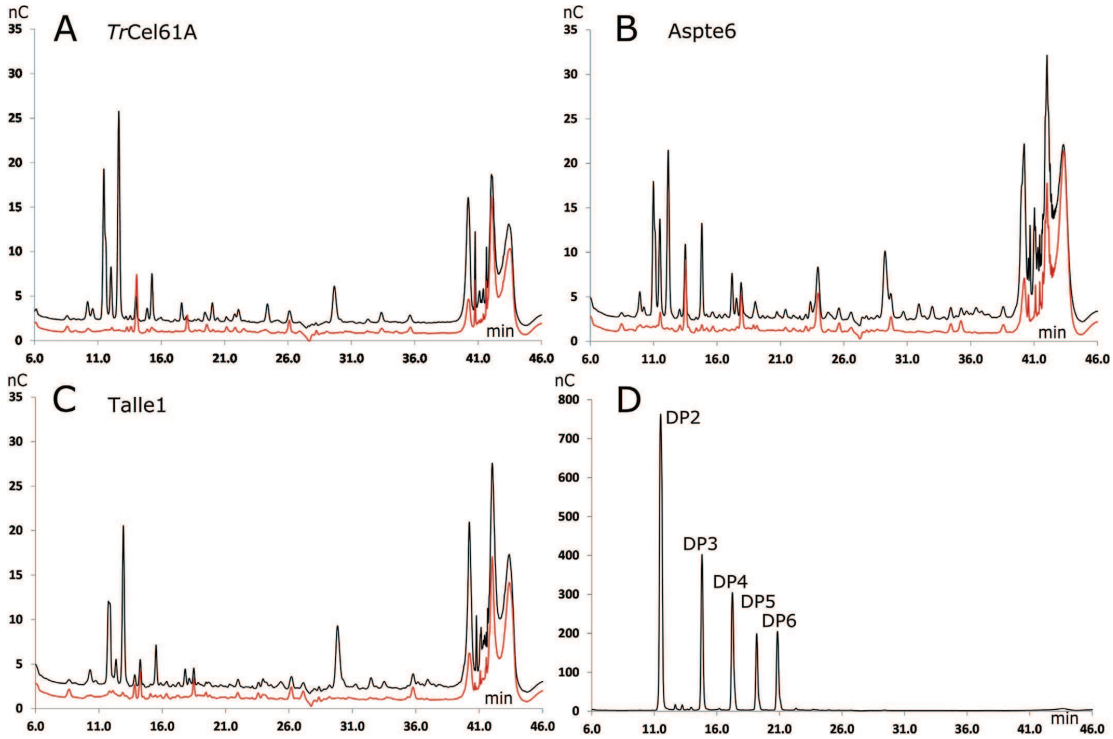

Fig. 2. HPAEC-PAD profile of LPMO treated NaOH pretreated soy spent flakes both with (black, 1 nC offset) and without (red) ascorbic acid as per Westereng et al. [34]. A: *Tr*Cel61A; B: Aspte6; C: Talle1; D: DP 2–6 cello-oligosaccharides. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

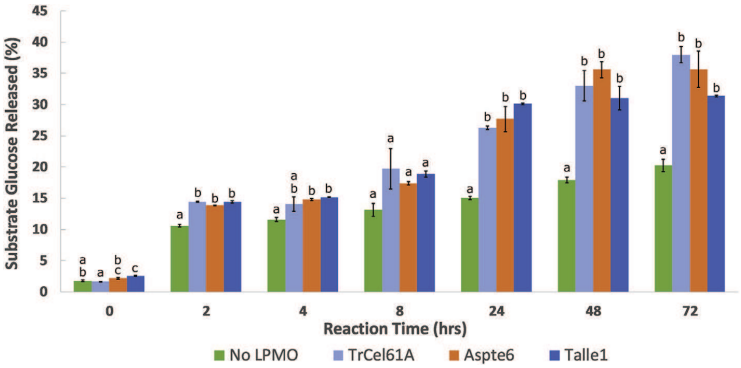

Fig. 3. Glucose release from NaOH pretreated soy spent flakes following treatment with LPMO (0.05 mg/mL protein), EG (0.05 mg/mL protein), and BG (0.125 mg/mL protein) in the presence of 1 mM ascorbic acid, as determined by HPAEC-PAD analysis. Data are shown as averages of duplicate analyses with standard deviations given by error bars. Tukey-Kramer HSD comparisons are provided for each time point with data points marked by differing letters at a given timepoint signifying statistically different means.

(*Tr*Cel61A, Aspte6, and Talle1) selected above show strong C4 oxidation, confirming the MS-MS results presented in Fig. 1. It is interesting to note that four LPMOs (Aspfu3, Aspte2, Aspte3, and Aspte4) showed evidence of C4 oxidation, but no activity on the pretreated spent flakes. Lastly, it should be noted that all oxidation effects described above were absent in control samples treated with each LPMO in the absence of ascorbic acid (results not shown).

2.3. Hydrolytic boosting effect between LPMO, endoglucanase, and beta-glucosidase on soy spent flakes

In our previous work, we demonstrated the hydrolytic boosting capability of *Tr*Cel61A when dosed in combination with EG for the enzymatic degradation of NaOH pretreated spent flakes [26]. In order to further explore this effect, the top-performing LPMOs described in Section 2.2 were evaluated for their hydrolytic boosting ability. Fig. 3 shows the HPAEC measured glucose release from NaOH pretreated soy spent flakes following LPMO treatment in combination with EG and BG for 0–72 h. We observe that all three of the LPMOs evaluated (*Tr*Cel61A, Aspte6, and Talle1) show a significant hydrolytic boosting effect when compared to samples treated without LPMO. Statistical evaluation by the Tukey-Kramer HSD method was conducted and the results are presented in Fig. 3. Additional statistical evaluation with two-way analysis of variance (ANOVA) describes the significant correlation between glucose release and both reaction time and LPMO treatment (Supplementary Information Section 5). This analysis confirms that the glucose release depends on the interaction of these factors, as evidenced by the data presented in Fig. 3. In addition to the Tukey-Kramer analysis presented in Fig. 3, additional Tukey pairwise comparisons were conducted on the complete dataset in Fig. 3 (irrespective of time point) and are provided in Supplementary Information Section 5. These results show that the LPMO treatments from 8 to 72 h result in statistically significant increases in glucose release over the controls lacking LPMO. In addition, differences between the boosting effects of the three enzyme treatments are not statistically significant at each of these time points. The addition of *Tr*Cel61A, Aspte6, and Talle1 result in glucose yield increases of 88%, 76%, and 55%, respectively, when compared to enzymatic treatment with EG and BG alone. *Tr*Cel61A and Aspte6 are capable of ~36% substrate glucose release after 72 h of reaction, the highest yields obtained in this study and a comparable result to those obtained in previous work with *Tr*Cel61A (34.48 ± 4.84%) [26]. It is also interesting to note the rate of reaction observed in Fig. 3. Significant glucose release is obtained within the first 2 h of reaction (10–15%), however no significant additional glucose release is observed from 2 to 4 h. After 24 h of reaction, the samples treated with LPMOs capable of hydrolytic boosting begin to significantly differentiate themselves from controls. While samples containing Talle1 showed the highest glucose release after 24 h, the reaction appears to have been completed in this timeframe as no additional glucose release was observed beyond this point.

In addition to evaluating the hydrolytic boosting activity of each LPMO alone, two-enzyme combinations of the top-performing LPMOs were also assessed. Fig. 4 shows the glucose release from NaOH pretreated spent flakes after 72 h incubation with these enzyme combinations. A one-way analysis of variance showed no statistically significant differences between these six treatments, indicating that the LPMO combinations tested are unable to increase the glucose release when compared with the individual LPMO treatments. Lastly, it should be noted that all hydrolytic

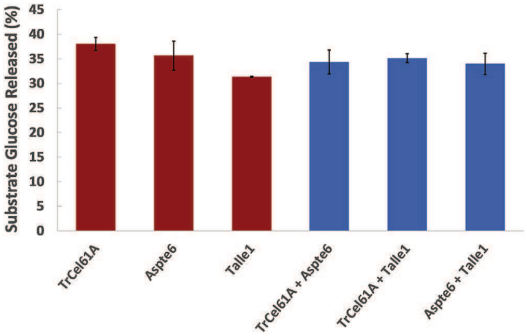

Fig. 4. Glucose release from NaOH pretreated soy spent flakes following 72 h treatment with LPMO(s) (0.05 mg/mL protein), EG (0.05 mg/mL protein), and BG (0.125 mg/mL protein) in the presence of 1 mM ascorbic acid, as determined by HPAEC-PAD analysis. Samples contain either a single LPMO (red) or two LPMOs (blue). Data are shown as averages of duplicate analyses with standard deviations given by error bars. One-way analysis of variance showed no statistically significant differences ($p > 0.05$) between the six treatments presented. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

boosting effects observed in Figs. 3 and 4 were not present in the absence of ascorbic acid (results not shown).

The data presented in Figs. 1 and 2 indicate that the three top-performing LPMOs from this study (*Tr*Cel61A, Aspte6, and Talle1) show similar oxidation on NaOH pretreated spent flakes. As described above, the hydrolytic boosting effect demonstrated in Fig. 3 is consistent across these three enzymes as well, with Fig. 4 showing that 50% of the LPMO dosage can be replaced with another similarly acting LPMO to result in comparable hydrolytic boosting effects. These results indicate that this is a more generalized effect, and not necessarily tied to the specific activity of an individual LPMO. However, the results presented above do show commonality between the LPMOs displaying activity on the pretreated spent flakes. *Tr*Cel61A, Aspte6, and Talle1 all show significant C4 oxidizing activity (Figs. 1 and 2) as well as activity on Avicel®, suggesting a link between these features and activity on pretreated spent flakes.

3. Conclusions

In this study, we have characterized the LPMO activity of twenty-four enzymes against four substrates: PASC, native soy spent flakes, NaOH pretreated soy spent flakes, and Avicel® PH-101 microcrystalline cellulose. Following expression in *T. reesei*, the enzymes were evaluated for the presence of N-terminal histidine and methylation of this histidine, with all enzymes showing these features. Twenty-one enzymes displayed oxidative activity on PASC, seven showed activity on Avicel®, seven showed activity on NaOH pretreated soy spent flakes, and none showed activity on native soy spent flakes. Of the seven LPMOs showing activity on pretreated soy spent flakes (*Tr*Cel61A, Chacr2, Aspfu1, Aspfu4, Aspfu5, Aspte6, and Talle1), three were selected as the top-performing enzymes (*Tr*Cel61A, Aspte6, and Talle1). ESI MS-MS fragmentation analysis demonstrated predominantly C4 oxidizing activity in these enzymes, and the presence of C4 oxidized compounds was confirmed with HPAEC analysis. These results show a link between an LPMO's C4 oxidizing ability, activity on the microcrystalline Avicel® substrate, and oxidative activity on pretreated soy spent flakes. This observed correlation between C4 oxidizing activity and activity on NaOH pretreated soy spent flakes demonstrates a link between substrate structure and LPMO oxidative regioselectivity. This observation may offer some insight into the reasons for the evolution of regioselectivity in LPMOs, and highlights the continued need for research in this area to more fully understand the reasons behind oxidative regioselectivity. In addition to these observations, the hydrolytic boosting effect of these enzymes towards the enzymatic degradation of pretreated spent flakes by EG and BG was evaluated. Two of the enzymes (*Tr*Cel61A and Aspte6) showed the most significant hydrolytic boosting ability, with release of 36% substrate glucose after 72 h compared with the 20% released in the absence of LPMO. Investigations into the glucose release with LPMO combinations showed no direct evidence of increased hydrolytic boosting when compared to treatment with individual LPMOs. The current results further expand our understanding of the ability of LPMOs to degrade this complex substrate, and open new and exciting opportunities for the application of these enzymes in the enzymatic degradation of soy polysaccharides and other industrially-relevant substrates in the future.

4. Materials and methods

4.1. Materials

Soy spent flakes were obtained from DuPont™ Nutrition & Health – Protein Solutions (St. Louis, MO) as a by-product of the soy protein isolate production process. The material was lyophilized and ground to pass a USA-Standard ASTM 40-mesh screen. Avicel® PH-101 microcrystalline cellulose, L-fucose (Fuc), L-rhamnose (Rha), D-arabinose (Ara), D-galactose (Gal), D-glucose (Glc), D-xylose (Xyl), D-glucuronic acid (GlcA), D-galacturonic acid (GalA), D-(+)-cellobiose, D-(+)-cellotriose, cellotetraose, cellopentaose, and cellohexaose were obtained from Sigma-Aldrich (St. Louis, MO). GE Healthcare PD MidiTrap G-25 columns were purchased from VWR (Radnor, PA). PASC was obtained from DuPont™ Industrial Biosciences (Palo Alto, CA) where it was prepared as described by Wood [35]. Invitrogen NuPAGE 4–12% Bis-Tris Gels, SeeBlue® Plus2 Pre-Stained Standard, and SimplyBlue™ SafeStain were obtained from Thermo Scientific (Waltham, MA).

4.2. Chemicals

Concentrated sodium hydroxide (50%; NaOH), 96% glacial acetic acid, copper(II) sulfate pentahydrate, methanol, acetonitrile, urea, ammonium bicarbonate, DL-dithiothreitol (DTT), iodoacetamide, formic acid, and trifluoroacetic acid (TFA) were all purchased from Sigma-Aldrich (St. Louis, MO). L(+)-ascorbic acid AnalaR NORMAPUR® was obtained from VWR (Radnor, PA). Sodium azide (NaN_3) was purchased from Merck Performance Materials (Darmstadt, Germany). The Bio-Rad Protein Assay Kit II (dye reagent and bovine serum albumin (BSA) standard) was obtained from Bio-Rad (Hercules, CA).

4.3. Enzymes

Twenty-four LPMOs (Table 1) were obtained from DuPont™ (Shanghai, China) following expression in a *Trichoderma reesei* (*Hypocrea jecorina*) strain deleted for the major cellulase genes and beta-glucosidase as described in PCT Pat. Appl. Publ. No. WO2009/048488[27]. Expression was conducted with the native leader peptide for each protein. The amino acid sequences, including the leader peptide sequences, are provided in FASTA format in Supplementary Information Section 1. Cell debris was removed by centrifugation and the supernatant was concentrated using a VivaFlow50 PES ultrafiltration membrane (MWCO 10 kDa) from Sartorius (Göttingen, Germany), with final protein concentrations (Table 1) determined as described in 4.5.3. The purity of the enzymes were assessed using SDS-PAGE gels (Supplementary Information Figure S1) as described in 4.5.4, and N-terminal characterization was performed as described in 4.5.5 to verify the expected presence of an N-terminal histidine with methylation of the imidazole [11]. Endo-1,4-β-D-glucanase from *Bacillus amyloliquefaciens* (EC 3.2.1.4; GH5) with 3500U activity (17.07 mg/mL protein) and beta-glucosidase from *Agrobacterium sp.* (EC 3.2.1.21; GH1) with 600U activity (2.48 mg/mL protein) were obtained from Megazyme International (County Wicklow, Ireland). Trypsin from bovine pancreas (EC 3.4.21.4), α-Chymotrypsin from bovine pancreas (EC 3.4.21.1), and endoproteinase Asp-N from *Pseudomonas fragi* (EC 3.4.24.33) were obtained from Sigma-Aldrich (St. Louis, MO).

4.4. Soy spent flake NaOH pretreatment

NaOH pretreated soy spent flakes were produced as described by Mittal, Katahira, Himmel & Johnson [32]. In brief, dry soy spent flakes were combined with 16.5% w/w NaOH in an amount of 8.57 mg per mL of 16.5% w/w NaOH. This mixture was stirred under nitrogen at 25 °C for 2 h. Upon completion, the material was separated by centrifugation and the soluble fraction was decanted off with the remaining insoluble fraction re-diluted in MilliQ water to the original reaction volume and vortexed. The centrifugation, separation and dilution described above were repeated until the pH

of the soluble fraction was neutral. The final, rinsed insoluble material was lyophilized and ground to pass a USA-Standard ASTM 40-mesh screen.

4.5. Material characterization

4.5.1. High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

The free monosaccharides in each sample were quantified by HPAEC-PAD using a Dionex ICS-3000 Ion Chromatography system with a CarboPac® PA100 guard column (50 × 2 mm) followed by a CarboPac® PA100 analytical column (250 × 2 mm) and a PAD, all from Dionex Corporation (Sunnyvale, CA). The flow rate used was 0.25 mL/min under the following elution profile, with MilliQ water as eluent A and 600 mM NaOH as eluent B: 0–18 min, 1.5% B; 18–19 min, 1.5–75% B; 19–29 min, 75% B; 29–29.1 min, 75–1.5% B; 29.1–43 min, 1.5% B. Monosaccharides were quantified using both external calibration standards containing a mixture of Fuc, Rha, Ara, Gal, Glc, Xyl, GlcA, and GalA, as well as variable internal calibration with Fuc added to each sample in the range of 6–13 ppm. The standard curves for each sugar were fit with a linear calibration curve without offset, except for Fuc where a second order polynomial calibration curve without offset was used.

HPAEC-PAD was also used for the analysis of the oxidation products of LPMO reactions using the method of Westereng et al. [34]. This involved the use of a Dionex ICS-3000 Ion Chromatography system with a CarboPac® PA1 analytical column (250 × 2 mm) and a PAD, all from Dionex Corporation (Sunnyvale, CA). Note that no guard column was used in these experiments. The flow rate used was 0.25 mL/min under the following elution profile, with 0.1 M NaOH as eluent A and 1 M NaOAc in 0.1 M NaOH as eluent B: 0–10min, 0–10% B; 10–35 min, 10–30% B; 35–40 min, 30–100% B (Dionex curve 6 - exponential); 40–41 min, 100-0% B; 41–50 min, 0% B.

4.5.2. Direct infusion electrospray ionization mass spectrometry (ESI-MS)

Mass spectrometry analysis by ESI-MS was performed on a linear ion trap LTQ Orbitrap Fusion from Thermo Scientific (Waltham, MA) coupled to a TriVersa NanoMate® chip-based electrospray device from Advion (Ithaca, NY). The analyses were done by direct infusion to the MS without chromatographic separation. Samples were prepared in a 96-well microtiter plate with a methanol:sample ratio of 1:2. The electrospray was controlled with Advion ChipSoft Manager version 8.3.1 and operated in positive mode with a gas pressure (N_2) of 0.6 psi, voltage of 1.8 kV, 5 μL tip collection, and sample temperature of 7 °C utilizing plastic tips (part no. 1004763) and a 5 μm ESI Chip® (part no. 1003446). Fluoranthene was used as an internal calibrant and the ion transfer tube temperature was set to 200 °C. The acquisition time was set to 2.0 min, with approximately 40 scans per minute for MS and MS-MS. MS full scans were performed in the m/z 300–1200 mass range, utilizing an Orbitrap detector at 120K resolution and 2 microscans. MS-MS scans were performed on Na-adducts with CID (collision-induced dissociation), using He as the colliding gas in the m/z 105–400 scan range with quadrupole isolation, Orbitrap detection at 15K resolution, an isolation window of 2, 50% collision energy, and 3 microscans. MS Data from ESI-MS was processed in Xcalibur 3.0.63 from Thermo Scientific (Waltham, MA).

4.5.3. Measurement of total protein concentration

Total protein concentration was measured using the Bio-Rad Colorimetric Protein Assay based on the Bradford method [36]. In brief, 50 μL of enzyme sample was combined with 2.5 mL of 4x diluted Protein Assay Dye Reagent Concentrate and incubated at room temperature for 5 min. Absorbance was measured at 595 nm and compared to the absorbance measured against known concentrations of BSA from 0.1 to 0.9 mg/mL. All measurements were performed in duplicate.

4.5.4. SDS-PAGE

SDS-PAGE gels were run using an Invitrogen XCell SureLock™ Mini-Cell Electrophoresis system, Invitrogen NuPAGE 4–12% Bis-Tris Gels, and SeeBlue® Plus2 Pre-Stained Standard from Thermo Scientific (Waltham, MA). Proteins were incubated at 90 °C for 5 min with 1.0 M sodium-phosphate buffer, pH 6 and sample buffer containing DTT and 1% bromophenol blue. Following electrophoresis, gels were stained with SimplyBlue™ SafeStain from Thermo Scientific (Waltham, MA).

4.5.5. N-terminal characterization with liquid chromatography-mass spectrometry (LC-MS)

Proteomic analysis was performed using a modified spin filter protease digestion as previously described by Manza et al. and Wiśniewski et al. [37,38]. Samples were reduced with DTT and alkylated with iodoacetamide prior to protease digestion. Amino acid sequences were used to select an optimal protease for N-terminal characterization: Chymotrypsin (Chacr1, Aspfu1, Aspfu2, Aspte2, Aspte5, Talle1, and *Tr*Cel61A), Trypsin (Aspfu3, Aspfu4, Aspfu5, Aspte6, Penci2, Aspte7, Aspte8, Malci2, Mycth1, and Mycth3), and Asp-N (Chacr2, Aspte1, Aspte3, Aspte4, Penci1, Malci1, and Mycth2). Following protease digestion, samples were dried in a Thermo Scientific (Waltham, MA) Savant™ SpeedVac™ at 45 °C for 8 h, and dissolved in 100 μL of 0.1% TFA for LC-MS analysis.

LC separation was performed using an UltiMate 3000 Nano LC system from Dionex Corporation (Sunnyvale, CA), a 5 μm Acclaim PepMap C18 guard column (20 mm × 100 μm) from Thermo Scientific (Waltham, MA), and a 3 μm ReproSil-Pur C18-AQ column (100 mm × 75 μm) from Dr. Maisch GmbH (Ammerbuch-Entringen, Germany). The flow rate used was 300 nL/min under the following elution profile, with H_2O/formic acid (100:0.1) as eluent A and acetonitrile/formic acid (100:0.1) as eluent B: 0–3 min, 2% B; 3–21 min, 2–41% B; 21–23 min, 41–95% B; 23–29 min, 95% B; 29–31 min, 95-2% B; 31–44 min, 2% B. MS and MS-MS analysis by ESI-MS was performed in positive mode on a linear ion trap LTQ Orbitrap Fusion with a capillary temperature of 200 °C, 2.1 kV ESI voltage, and quadrupole isolation. For MS-MS analysis, both CID (collision-induced dissociation) and HCD (higher-energy collisional dissociation) fragmentation were performed. MS data was processed in Xcalibur 3.0.63 from Thermo Scientific (Waltham, MA) and Mascot Server from Matrix Science Inc. (Boston, MA). A summary of the results from this analysis is provided in Supplementary Information Section 3.

4.6. Enzymatic reactions

4.6.1. Primary LPMO screening

Reaction mixtures of 200 μL total volume contained 4 mg/mL substrate cellulose, 0.02% sodium azide, and 1 mM ascorbic acid in 10 mM Na-acetate, pH 6.0. All LPMOs were Cu(II) saturated by incubating 0.3 mg/mL protein in 10 mM Na-acetate, pH 6.0 with a 3-fold molar excess of $Cu(II)SO_4$ for 30 min at room temperature. Excess copper was removed by PD MidiTrap G-25 filtration, as described by Loose, Forsberg, Fraaije, Eijsink, & Vaaje-Kolstad [39] with 10 mM Na-acetate, pH 6.0 used in place of the 20 mM Tris-HCl, pH 8.0. LPMOs were dosed based on protein content at 0.2 mg/mL. Samples were incubated at 40 °C with 600 rpm mixing for 24 h. Following the reaction, all enzyme samples were heat-

inactivated through incubation at 95 °C for 5 min and centrifuged at 16,300 × *g* for 20 min to isolate the supernatants from the pellets.

4.6.2. Glucose release with LPMO, endoglucanase, and beta-glucosidase

All LPMOs were Cu(II) saturated as described in 4.6.1, however a protein concentration of 0.2 mg/mL was used during copper saturation. LPMOs were dosed based on protein content at 0.05 mg/mL with samples incubated at 40 °C with 600 rpm mixing for varying times from 0 to 72 h. For reactions utilizing a combination of LPMOs, the total LPMO protein dosage was divided equally, with the total maintained at 0.05 mg/mL. All reactions also contained endo-1,4-β-D-glucanase (EG) and beta-glucosidase (BG) dosed at 0.05 and 0.125 mg/mL, respectively. Following the reaction, all enzyme samples were heat-inactivated through incubation at 95 °C for 5 min and centrifuged at 16,300 × *g* for 20 min to isolate the supernatants from the pellets. Glucose release was quantified by HPAEC-PAD as described in 4.5.1 and sample data was converted into substrate glucose released (%) by dividing the amount of glucose released in the enzyme treatment by the total amount present in the original reaction substrate and multiplying by 100. Glucose release was compared in Minitab® 17 from Minitab Inc. (State College, PA) using two-way analysis of variance and means comparisons by the Tukey pairwise comparison method.

4.6.3. Beta-glucosidase treatment of LPMO reaction products

Following LPMO enzymatic treatment, supernatants were treated with beta-glucosidase from *Agrobacterium sp*. to degrade non-C4 oxidized reaction products for analysis with HPAEC-PAD as described in 4.5.1. Beta-glucosidase was dosed based on protein at 0.05 mg/mL in 100 μL reactions containing 40% sample from the primary enzymatic treatment in 10 mM Na-acetate buffer, pH 6.0. Samples were incubated at 40 °C with 600 rpm shaking for 20 h. Following the reaction, beta-glucosidase was heat-inactivated by incubating the samples at 95 °C for 5 min and samples were analyzed by HPAEC-PAD using the method of Westereng et al. [34].

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The author contributions are as follows: B. C. P. contributed to the study design, analysis and interpretation of the results as well as the writing of the manuscript. J. W. A. contributed to the study design, data interpretation, and manuscript revision. Z. Z. conducted the enzyme expression and production for the samples utilized in this study. J. W. and A. S. M. contributed to the study design, data interpretation, manuscript revision, and project supervision.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.carres.2017.07.004.

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